2009

FACTORS INFLUENCING PLACENTAL TRANSFER OF LOPINAVIR: BINDING, UPTAKE AND EFFLUX

Abhishek Gulati
Virginia Commonwealth University

School of Pharmacy
Virginia Commonwealth University

This is to certify that the thesis prepared by Abhishek Gulati entitled
FACTORS INFLUENCING PLACENTAL TRANSFER OF LOPINAVIR:
BINDING, UPTAKE AND EFFLUX
has been approved by his committee as satisfactory completion of the thesis requirement
for the degree of Master of Science in Pharmaceutical Sciences

Phillip M. Gerk, Pharm D., Ph.D., School of Pharmacy

F. Douglas Boudinot, Ph.D., School of Pharmacy

Scott W. Walsh, Ph.D., School of Medicine

Douglas H. Sweet, Ph.D., School of Pharmacy

Peter R. Byron, Ph.D., Chairman of the Department of Pharmaceutics, School of Pharmacy

Victor A. Yanchick, Ph.D., Dean of the School of Pharmacy

Dr. F. Douglas Boudinot, Ph.D., Dean of the Graduate School

June 29, 2009

FACTORS INFLUENCING PLACENTAL TRANSFER OF LOPINAVIR: BINDING, UPTAKE AND EFFLUX

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

by

ABHISHEK GULATI
Bachelor of Pharmacy, Jamia Hamdard University, India, 2003
Master of Pharmacy (Pharmaceutics), Punjabi University, India, 2005

Director: PHILLIP M. GERK, Pharm.D., Ph.D.
Assistant Professor, Department of Pharmaceutics

Virginia Commonwealth University
Richmond, Virginia
June 2009

Dedicated to my Respected Parents,

my brother Amit

and

my loving wife, Puja

Acknowledgements

A journey is easier when you travel together. Interdependence is certainly more valuable than independence. This thesis is the result of research work whereby I have been accompanied and supported by many people. It is a pleasant aspect that I have now the opportunity to express my gratitude for all of them.

I would like to start this chain of my gratitude by mentioning the names of the most important persons in my life, my parents, **Mr. Anil Gulati** and **Mrs. Kulbeer Gulati**. Words prove a meager media to write down my feelings for them. Their love and support will remain indelible forever in my life. No words acknowledge the love and support I have received from my brother, **Dr. Amit Gulati**. I thank him for encouraging me all my life. I am extremely fortunate in having the best of family that God could provide on this earth.

I would like to thank my mentor and advisor, Dr. Phillip M. Gerk for all his enthusiastic support during my time here at VCU. His scientific inputs and patient instructions always helped me. Thanks for all his genuine support whenever asked for.

I owe my special thanks to Dr. Douglas Boudinot for all his help with the protein binding studies. His expertise in the field of pharmacokinetics helped me learn a lot and has boosted my interest in the field. His inputs and suggestions have been a very important part in completing this research work.

I also owe special thanks to Drs. Scott W. Walsh, Douglas Sweet and Jürgen Venitz for their suggestions and words of advice. Dr. Walsh's expertise on placental physiology, Dr. Sweet's valuable suggestions with the BeWo cell culture model and Dr. Venitz's inputs during the PK/PD/DT research group meetings and otherwise helped me accomplish this work.

I acknowledge the financial support for this project from National Institute of Health Grant 1P60-MD002256 and the Higher Education Equipment Trust Fund of Virginia. I also thank VCU School of Pharmacy, Department of Pharmaceutics for the financial support for my graduate education. I would also like to mention the name of Dr. Kenneth Audus (The University of Kansas Medical Center, Kansas City, KS) for providing us with the BeWo cells and also to Pallabi Mitra for her help in growing the cells.

I would like to acknowledge the support given to me by my friends and seniors who at all junctions made me cheerful and reduced my tensions from time to time. I would like to specially mention the names of Dave, Soniya, Satjit, Pravin, Lokesh, Kumar, Taghreed, Bhawana, Priya, Megha, Prajakta and Swati for their encouragement at different phases of this thesis.

I would also like to thank a lot to God, with whose grace and beatitude, I moved through this venture.

At the last and most important I thank a lot to my wife, **Puja**, for her support during the past years. She was a distant companion in all my ups and downs in the Graduate Program here at VCU. I acknowledge all the encouragement and love offered by her.

Table of Contents

List of Tables

Page

List of Figures

Page

List of Abbreviations

AAG	α_1-acid glycoprotein
ABC	ATP-binding cassette
ABCB1	ATP-binding cassette transporter isoform B1; P-glycoprotein (P-gp), Multidrug Resistance 1 (MDR1)
ABCB4	ATP-binding cassette transporter isoform B4; Multidrug Resistance 3 (MDR3)
ABCC1	ATP-binding cassette transporter isoform C1; Multidrug resistance protein 1 (MRP1)
ABCC11	ATP-binding cassette transporter isoform C11; Multidrug resistance protein 8 (MRP8)
ABCC2	ATP-binding cassette transporter isoform C2; Multidrug resistance protein 2 (MRP2)
ABCC3	ATP-binding cassette transporter isoform C3; Multidrug resistance protein 3 (MRP3)
ABCC4	ATP-binding cassette transporter isoform C4; Multidrug resistance protein 4 (MRP4)
ABCC5	ATP-binding cassette transporter isoform C5; Multidrug resistance protein 5 (MRP5)
ABCC7	ATP-binding cassette transporter isoform C7; Multidrug resistance protein 7 (MRP7)
ABCG2	ATP-binding cassette transporter isoform G2; Breast cancer resistance protein (BCRP)
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCRP	Breast cancer resistance protein (human isoform)
Bcrp1	Breast cancer resistance protein 1 (rodent ortholog)
BMI	Body mass index
BSA	Bovine serum albumin

BSP	Bromosulfophthalein
CYP	Cytochrome P450
CYP3A4	Cytochrome P450 isoform 3A4
DMEM	Dulbecco's modified Eagle's medium
DNP-SG	2, 4-dinitrophenyl-S-glutathione
DPBS	Dulbecco's Phosphate Buffered Saline
EDTA	Ethylene diamine tetra-acetic acid
EMS	Ethyl methanesulfonate
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GSH	Reduced glutathione
HAART	Highly Active Anti-Retroviral Therapy
hCC	Human choriocarcinoma cell lines
HEK	Human embryonic kidney
HIV	Human immunodeficiency virus
hL	Human liver
hPl	Human placenta
HSA	Human serum albumin
hTCs	Human trophoblast cells
hTr	Human trophoblast cells
MDCK	Madin-Darby canine kidney

MDR1	Multidrug resistance 1 (human isoform)
MDR3	Multidrug resistance 3 (human isoform)
MRP	Multidrug resistance protein (human isoform)
MRP8	Multidrug resistance associated protein 8 (human isoform)
OATP	Organic anion transporting polypeptides
OATP1A2	Organic anion transporting polypeptide (human) isoform 1A2
OATP1B1	Organic anion transporting polypeptide (human) isoform 1B1
OATP1B3	Organic anion transporting polypeptide (human) isoform 1B3
OATP2B1	Organic anion transporting polypeptide (human) isoform 2B1
OATP3A1	Organic anion transporting polypeptide (human) isoform 3A1
OATP4A1	Organic anion transporting polypeptide (human) isoform 4A1
PBMC	Peripheral blood mononuclear cell
PBS	phosphate buffered saline
P-gp	P-glycoprotein
PI	Protease inhibitor
RED	Rapid equilibrium dialysis
Rho123	Rhodamine 123
RT-PCR	Reverse transcription polymerase chain reaction
SLCO	Solute carrier organic anion transporter superfamily
TC	Taurocholate

Abstract

FACTORS INFLUENCING PLACENTAL TRANSFER OF LOPINAVIR: BINDING, UPTAKE AND EFFLUX

By Abhishek Gulati, B. Pharm., M. Pharm.

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

Major Director: Phillip M. Gerk, Pharm.D., Ph.D.
Assistant Professor, Department of Pharmaceutics

HIV protease inhibitors are an important component of Highly Active Antiretroviral Therapy used to treat HIV infected pregnant women. They have a low placental transfer and are highly plasma protein bound. The purpose of this thesis was to characterize the factors limiting placental passage and fetal exposure to lopinavir. These factors include lopinavir plasma protein binding and uptake, cellular binding, and efflux of lopinavir in the placental trophoblast cells.

First, we determined the unbound fraction of lopinavir in cord blood and characterized the binding of lopinavir to α_1-acid glycoprotein (AAG) and human serum

albumin (HSA), and displacement by ritonavir. Serum was obtained from cord blood from placentae obtained after cesarean section of healthy non-HIV infected women (n=4). The unbound fraction of lopinavir in serum obtained from this cord blood was 0.02 ± 0.01. The unbound fraction of lopinavir in separately obtained maternal serum samples (n=4) was 0.009 ± 0.001, which was not significantly different from that observed with cord serum samples. Varying concentrations of lopinavir, AAG, and HSA in buffer solutions were then used to characterize the lopinavir binding. The data were fit to obtain the number of binding sites (N) and equilibrium dissociation constant (K_D). Binding of lopinavir to AAG (7-23 μM) was saturable with K_D of 5.0 ± 1.1 μM and N of 1.2 ± 0.2. At low HSA concentrations (15-152 μM), lopinavir binding K_D was 24.3 ± 8.7 μM and N was 1.1 ± 0.4; however at 758 μM, lopinavir binding was essentially unsaturable. Additionally, lopinavir binding to AAG and HSA was not sensitive to ritonavir within the range of therapeutic concentrations.

Next, we examined lopinavir uptake, binding and efflux using the BeWo human trophoblast cell culture model. BeWo cells were treated with ^{3}H-lopinavir in the absence or presence of inhibitors of ATP- Binding Cassette transporters. The radioactivity was then measured in the buffer and the cells after incubating for different time intervals and at two temperatures. Verapamil (100μM) stimulated apparent efflux of ^{3}H-lopinavir by two fold, possibly due to ABCC2. In addition, this efflux process was 75% inhibited by reduced temperature (4°C). Ritonavir (10 μM) also stimulated ^{3}H-lopinavir efflux, whereas GF120918 (1 μM) had no effect. Reduced temperature (4°C), verapamil (100 μM) or ritonavir (10 μM) individually did not significantly affect the binding of ^{3}H-lopinavir to

cell homogenates. However, slight but significant binding displacement by verapamil at 4°C was observed. ^{3}H-lopinavir uptake was not sensitive to verapamil, bromosulfophthalein, taurocholate or to reduced temperature suggesting uptake involves diffusion rather than Organic Anion Transporting Polypeptide transporters. The results suggested that interplay between cellular binding and ABC efflux transporters, in addition to simple diffusion, determines the extent of ^{3}H-lopinavir distribution into BeWo cells.

CHAPTER 1 INTRODUCTION

The placenta is a complex organ that forms an interface between the maternal and the fetal circulation during the gestation period. It functions in the exchange of nutrients, respiratory gases, and metabolic waste, and is also a source of hormones. Additionally, it may limit fetal exposure to various xenobiotics in the maternal circulation. Drug concentrations achieved in the fetus depend on the maternal drug concentration, kinetics of drug transfer across the placenta, and placental and fetal drug metabolism. The rate and extent of drug transfer depends on physicochemical properties of the drugs and the physiological characteristics of the maternal-placental-fetal unit (Syme et al., 2004). The properties of drugs that may affect the extent of placental transfer include their molecular weight, pKa, lipid solubility, and plasma protein binding. Also, several types of uptake and efflux transporters are present on the maternal facing (brush-border or apical) as well as the fetal facing (basolateral) side of syncytiotrophoblast, which potentially modulate the placental passage of drugs intended to treat the mother, the fetus or both. Therefore, these transporters may limit the passage of some drugs intended for maternal or fetal treatment and thus, alter drug exposure. Among them are the ATP Binding Cassette (ABC) and the Organic Anion Transporting Polypeptide (OATP) transporters. ABC transporters are efflux transporters that utilize the energy of ATP hydrolysis to transfer substances out of the cells,

usually toward maternal circulation. OATPs are plasma membrane transport proteins that mediate the active cellular uptake of a variety of compounds.

Pregnancy results in physiologic changes that not only concern the mother but also the fetus and the placenta. Besides changes observed throughout the pregnancy in the cardiovascular system, respiratory system, GI system, kidneys, and the uterus, there are a number of significant changes that occur in the placenta during gestation. Some of these physiological changes are summarized below in Table 1 (Loebstein et al., 1997; Anderson, 2005). These physiological changes, which may affect all four pharmacokinetic processes, may result in altered drug concentrations during pregnancy. These changes may also affect the expression of various transporters present in the placenta and as a result, may affect the extent of placental drug transfer.

Table 1: Effect of physiological changes observed during pregnancy on pharmacokinetics, in general (Loebstein et al., 1997; Anderson, 2005).

ABSORPTION

- Reduction in gastric acid secretion causes an increase in gastric pH
- Decrease in intestinal motility causes an increase in gastric emptying time and intestinal transit time
- Nausea and vomiting, which are pronounced during the earlier stages of pregnancy, may also affect drug absorption

DISTRIBUTION

- Total body water, plasma volume and body fat stores also increase and may affect drug distribution
- Plasma protein concentrations also change during pregnancy. Albumin concentrations decrease whereas concentrations of α-acid glycoprotein are equivocal.
- Volume of distribution increases which causes a decrease in peak drug concentrations

METABOLISM

- Estrogen and progesterone levels increase, which may affect the activity and expression of many metabolizing enzymes. Activity of CYP3A4, 2D6, 2C9 and 2A6 increases whereas that of CYP1A2 and 2C19 decreases during pregnancy

EXCRETION

- Renal plasma flow and glomerular filtration rate increase, which increases the renal clearance of a drug
- Activity and expression of different transporters are also altered

HIGHLY ACTIVE ANTIRETROVIRAL THERAPY DURING PREGNANCY

Human Immunodeficiency Virus (HIV) - infected patients are defined as having the Acquired Immunodeficiency Syndrome (AIDS) if they have CD4 cell count of less than 200/mm^3. Since the onset of the epidemic, close to 60 million people have been infected with the virus, with almost 20 million dying from its complications. Of the more than 40 million people with HIV infection today, close to half of them are women, and more than 3 million are children under the age of 15 (Libman and Makadon, 2007). To treat HIV infected patients, multiple antiretroviral drugs are used in what is known as Highly Active Antiretroviral Therapy (HAART). Over the past decade, HAART has led to a significant increase in life expectancy, at least in drug-naïve patients. Recent studies with newly discovered agents have resulted in an extension of this benefit to treatment-experienced patients as well, with over 60% of the subjects in a study achieving maximal virologic suppression when treated with raltegravir, the first drug of a new class of HIV drugs, the integrase inhibitors. According to the authors, its combination with darunavir and etravirine may extend the benefit to over 90% of the patients. Similar results may also be expected with maraviroc, a newly developed chemokine receptor antagonist (Conway, 2009).

In pregnant HIV infected patients, however, HAART serves two goals, providing adequate treatment for the mother and preventing viral transmission to the fetus. The use of HAART regimens has led to a significant reduction in occurrence of perinatal transmission to less than 2% (Watts, 2006). The use of different drug combinations with the inclusion of novel compounds may, however, result in unacceptable side effects in clinical practice that

may not have been detectable during clinical trials. These have not only increased the chances of short-term toxicities but also long-term impact on the mother and the child. Because of toxicities observed after use of these antiretroviral drugs in pregnant women, there have been guidelines published for use of these drugs in pregnant HIV-infected women (http://aidsinfo.nih.gov/ContentFiles/PerinatalGL.pdf). Based on the clinical data available, NIH has divided the antiretroviral drugs into four categories for their use in pregnant patients: recommended agents, alternate agents, drugs for use in special circumstances, and drugs for which there is insufficient data to recommend use. According to 2009 guidelines, lopinavir/ ritonavir is the only recommended agent in the category of protease inhibitors for use in HIV-infected pregnant patients (http://aidsinfo.nih.gov/ContentFiles/PerinatalGL.pdf).

As mentioned earlier, different physiological changes that occur during pregnancy may also alter the pharmacokinetics of different drugs. In pregnancy, the concentrations of nucleoside and non-nucleoside reverse transcriptase inhibitors may not change significantly but concentrations of protease inhibitors (PI) are significantly reduced (Marzolini and Kim, 2005). As protease inhibitors are an integral part of HAART, lower concentrations may result in treatment failure and poor therapeutic outcomes. Thus, the observed physiological changes during pregnancy may necessitate dose adjustments to achieve therapeutic concentrations in pregnant women.

There are various ABC transporters that are expressed in the placenta, of which HIV protease inhibitors are known to be substrates of many of them. Most of the HIV protease inhibitors are administered with ritonavir, which acts to inhibit excretion mediated

by ABCB1 and metabolism mediated by CYP3A4, thus causing an increase in concentrations of the HIV protease inhibitors (Huisman et al., 2001). The drug-transporter interactions together with a change in expression of these transporters that may occur during pregnancy, there may be an affect on the therapeutic outcome. Thus, there is a need to know which transporters and to what extent they are expressed in the placenta.

EXPRESSION OF ABC TRANSPORTERS IN THE PLACENTA

ABCB1 (P-glycoprotein):

The expression, localization and function of P-glycoprotein (P-gp) in the placenta have been reviewed (Ceckova-Novotna et al., 2006). P-gp, encoded by the multi drug resistance 1 (MDR1/ ABCB1) gene, is present on the apical (brush-border) syncytiotrophoblast membrane and actively extrudes many substrates, including a wide range of drugs. The efflux of lipophilic or slightly charged compounds from the cell takes place with the utilization of energy from ATP hydrolysis. Rhodamine 123 (Rho123), a well known ABCB1 substrate, has a higher maternal-to-fetal transplacental passage in the presence of the ABCB1 inhibitors PSC833, cyclosporine A, quinidine, and chlorpromazine. The fetal-to-maternal passage of Rho123 decreases in the presence of PSC833, cyclosporine A, and quinidine (Pavek et al., 2003). These data indicate that the transplacental passage of Rho123 is in part directed by ABCB1. Atkinson et al. carried out RT-PCR and western blotting studies in placenta, primary cytotrophoblast cell cultures and BeWo, JAr, and JEG choriocarcinoma cell lines (Atkinson et al., 2003). They found ABCB1 to be absent or expressed minimally in BeWo and JEG cell lines (antibodies used

were clone F4 or C219). In the syncytiotrophoblast, ABCB1 was present predominantly on the apical side (maternal-facing). Western blotting analysis carried out by Nagashige et al., with human placental membrane vesicles indicated the localization of ABCB1 on the apical membrane (Nagashige et al., 2003). The primary antibody used by the authors against ABCB1 was C219. In other studies, no ABCB1 expression was revealed at the mRNA and protein levels (antibody used – C219 anti-ABCB1 monoclonal antibody) in BeWo cells (Magnarin et al., 2008). The low level or absence of ABCB1 expression in BeWo cells observed by these authors was contrary to the higher level observed by others (Ushigome et al., 2000; Utoguchi et al., 2000). The antibodies used by these authors were mouse monoclonal antibody C219 or P170-glycoprotein, multidrug-resistance-related clone JSB-1 (Ushigome et al., 2000) and antihuman C219 monoclonal antibody (Utoguchi et al., 2000); however C219 antibody also recognizes MDR3 (ABCB4) which may have resulted in a false positive result for ABCB1 expression. Ceckova-Novotna et al. mention that expression of ABCB1 seemed to differ among particular clones of BeWo cells, as they also observed an undetectable expression of ABCB1 mRNA when they used BeWo cells from the same source as Atkinson et al. (Ceckova-Novotna et al., 2006). In contrast to ABCB1, MDR3/ABCB4 gene product transports a limited number of substrates. The expression of ABCB4 in human placenta has been reported (Patel et al., 2003); however, its functional significance in the placenta is unknown. It is present in the syncytial basolateral membrane and cells lining the fetal capillaries (Evseenko et al., 2006b), and is upregulated four fold in third trimester placentae compared to first trimester (Patel et al.,

2003), in contrast to ABCB1, which is expressed more abundantly during the early gestation (Sun et al., 2006).

ABCG2 (Breast Cancer Resistance Protein):

Breast Cancer Resistance Protein (BCRP, ABCG2) confers resistance to hydrophobic/ anionic xenobiotics like mitoxantrone, topotecan derivatives, and anthracyclines. It is present on the apical side of the syncytiotrophoblast and its substrates include a wide variety of anticancer agents, organic cations and lipophilic conjugates (Evseenko et al., 2006a). ABCG2 is known as a "half-transporter" as it is reported to be composed of just one transmembrane region and one ATP-binding domain compared to other ABC transporters which are composed of two transmembrane regions and two ATP-binding domains (Brangi et al., 1999), although the active form is thought to be a dimer. Mao et al. have reviewed the role of ABCG2 in multi-drug resistance and drug disposition (Mao and Unadkat, 2005). Expression of ABCG2 in BeWo cells (antibody used – monoclonal anti-ABCG2 antibody – clone BXP-21) has been reported (Magnarin et al., 2008). Functional evidence of Bcrp1 activity in the mouse placenta was established by Jonker et al. (Jonker et al., 2000). They showed that treatment with a ABCG2 inhibitor GF120918, which also inhibits ABCB1, decreases plasma clearance and hepatobiliary excretion of topotecan and increases absorption of this anticancer drug from the small intestine in ABCB1 knockout mice. In pregnant GF120918 treated ABCB1 deficient mice, the relative fetal concentration of topotecan was found to be 2-fold higher than that in pregnant vehicle treated mice (Jonker et al., 2000). The authors explained that topotecan

was a weaker substrate for ABCB1 and suggested the increase in bioavailability was due to inhibition of ABCG2 by GF120918, resulting in increased intestinal absorption and decreased biliary excretion of the agent. Also, inhibition of ABCG2 in the placenta may have resulted in higher fetal topotecan concentrations.

ABCC family (Multidrug Resistance Proteins):

Evseenko et al. have reviewed different ABC transporters involved in the active transport across the human placenta (Evseenko et al., 2006a). The multidrug resistance protein (MRP) family of drug transporters has nine members. They are organic anion transporters; however, ABCC1, ABCC2, and ABCC3 also transport certain neutral drugs. Their substrate range is much narrower compared to ABCB1, and are involved in the efflux of toxic fetal metabolites, in particular unconjugated bilirubin and bile acids. They are also involved in the removal of glutathiolated, glucuronidated, or sulfated metabolites from cells. St-Pierre et al. examined if members of the ABCC family were expressed in term placenta (St-Pierre et al., 2000). Immunofluorescence and immunoblotting studies carried out by the authors showed that ABCC2 was localized to the apical syncytiotrophoblast membrane, where as ABCC1 and ABCC3 were predominantly expressed in blood vessel endothelia with some evidence for expression in the apical syncytiotrophoblast (St-Pierre et al., 2000). However, ABCC1 is also reported to be localized on the basolateral side of the placental membrane. Atkinson et al. carried out RT-PCR and western blotting studies in placenta, primary cytotrophoblast cell cultures and BeWo, JAr, and JEG choriocarcinoma cell lines, and found ABCC1 to be expressed

ubiquitously (Atkinson et al., 2003). In the syncytiotrophoblast, ABCC1 was present on the basolateral side. Western blotting analysis carried out by Nagashige et al., with human placental membrane vesicles indicated the localization of ABCC1 on the basolateral membrane (Nagashige et al., 2003). Functional evidence for apical localization of ABCC1 has been observed by our laboratory (Vaidya et al., 2009). These studies indicated the involvement of an apical ABC transporter that was inhibited by MK571, dipyridamole, and verapamil in the efflux of 2, 4-dinitrophenyl-S-glutathione (DNP-SG) from the villous tissue. Their results were consistent with their ^{3}H-DNP-SG transport data with Sf9 membrane vesicle experiments. The authors supported this by explaining that in cultured placental villous tissue model, the fetal capillaries are collapsed and basolateral DNP-SG efflux would not be expected. Histological examination of the villous tissue revealed that fetal capillary endothelial vessels were not exposed to the medium, thus supporting the argument of apical localization of ABCC1 (Vaidya et al., 2009). In BeWo cells, expression of ABCC1 (antibody used – A23 anti-ABCC1 polyclonal antibody) has been reported in other studies, however, the authors did not discuss the localization of the transporter (Magnarin et al., 2008). Pascolo et al. compared the expression of ABCC1, ABCC2, ABCC3, and ABCC5 in human placental tissue and in BeWo cells by real-time RT-PCR analysis (Pascolo et al., 2003). They expressed the protein expression by Western blot. The authors found ABCC1 and ABCC3 to be most abundantly expressed in the placenta. Only ABCC1 was highly expressed in BeWo cells compared to ABCC2, ABCC3, and ABCC5 that were weakly expressed (Pascolo et al., 2003). Langmann et al. carried out real-time reverse transcription-PCR expression profiling of the complete human ABC transporter

superfamily in various tissues and have documented the ABCC4, -5, and -7 mRNA expression in the placenta in addition to the above mentioned ABCC transporters (Langmann et al., 2003). Schuetz et al. have investigated if overexpression and amplification of the ABCC4 gene was correlated with ATP-dependent efflux of some dideoxynucleosides, which are a common component of HAART (Schuetz et al., 1999). The two were found to be correlated and overexpression of ABCC4 mRNA and protein severely impaired the antiviral efficacy of the studied reverse transcriptase inhibitor drugs. Higher resistance to the studied nucleoside monophosphate analogs and amplification of the ABCC4 gene was correlated with enhanced drug efflux (Schuetz et al., 1999). ABCC5 mRNA as well as protein expression in the human placenta has been reported and its functional activity has been measured in the placental basal membrane vesicles and fetal endothelium (Meyer Zu Schwabedissen et al., 2005). However, localization and physiological role of ABCC7 in pregnancy still remains obscure (Evseenko et al., 2006a). Expression of ABCC11 (MRP8) is also reported in the placenta (Tammur et al., 2001; Yabuuchi et al., 2001). Its substrate specificity appears as broad as that of ABCC1 and ABCC2. Although the ABCC family plays important roles in other barrier epithelia, their impact and role in the human placenta is not as clearly established.

HIV PROTEASE INHIBITORS AND ABC TRANSPORTERS

HIV protease inhibitors, as substrates of ABC transporters, have been the targets of many studies that have been carried out to see the effect of pharmacokinetic changes during pregnancy, on the placental transfer of this category of drugs. The literature shows

several cases in which efflux transporters are responsible for low drug concentrations reaching the fetus. For example, a deficiency in mouse placental ABCB1 has been shown to enhance fetal susceptibility to chemically induced birth defects by avermectins (Lankas et al., 1998). Similarly, Smit et al. showed that 2.4-, 7-, or 16-fold more [^{3}H]-digoxin, [^{14}C]-saquinavir, or paclitaxel, respectively, entered the ABCB1 deficient Abcb1a$^{-/-}$/1b$^{-/-}$ fetuses than entered wild type fetuses in the study carried out in mice (Smit et al., 1999). They also used the ABCB1 inhibitors PSC833 or GG918 to show that blocking ABCB1 using these inhibitors resulted in increased transplacental passage of these drugs into the fetus. Molsa et al. observed similar results in studies carried out with human placentae, in which preperfusion with PSC833 increased the maternal-to-fetal transfer of saquinavir by 7.9-fold (0.18% ± 0.09% vs 1.4% ± 0.67%), and preperfusion with GG918 increased it by 6.2-fold (0.18% ± 0.09% vs 1.1% ± 0.39%) (Molsa et al., 2005). The authors also observed 108-fold higher saquinavir transfer in the fetal-to-maternal direction than from maternal to fetal direction (0.18% ± 0.09% vs 19.5% ± 14.5%). PSC833 did not affect saquinavir transfer in the fetal-to-maternal direction (16.6% ± 14.2% vs 19.5% ± 14.5%), possibly because it was already relatively high. The authors also found that ABCB1 expression was correlated with PSC833-induced change in saquinavir transfer, but did not observe an effect of ABCB1 polymorphism on the PSC833- or GG918-induced change in saquinavir transfer in a small number of samples (Molsa et al., 2005).

The interaction of HIV protease inhibitors with ABCB1 has been reported (Srinivas et al., 1998). At a concentration of 5 μM, ritonavir, nelfinavir, and indinavir significantly increased calcein fluorescence in CEM/ VBL100 cells in the calcein-AM assay, indicating

ABCB1 was inhibited. However, at a higher concentration of 50 μM, saquinavir was also observed to have an effect. This observed increase in calcein fluorescence was probably due to a decreased ABCB1 activity caused by the HIV protease inhibitors used in the study. The drugs caused a decrease in calcein-AM efflux, which was seen as an increased fluorescence. Thus, all four protease inhibitors studied were found to interact with ABCB1 with affinities in the order ritonavir > nelfinavir > indinavir > saquinavir (Srinivas et al., 1998). In the same study, all four drugs were also found to increase the calcein fluorescence in $ABCC1^{+}$ CEM/VM-1-5 cells suggesting inhibition of ABCC1 by HIV protease inhibitors (Srinivas et al., 1998).

In several different studies, ritonavir, saquinavir, indinavir and nelfinavir were observed to inhibit ABCB1 (Srinivas et al., 1998; Gutmann et al., 1999; Profit et al., 1999; Choo et al., 2000; Miller et al., 2000; Washington et al., 2000; Huisman et al., 2001). Ritonavir was the most potent of them. In a study by Drewe et al., ritonavir (IC_{50} = 0.2 μM) was found to be a more potent ABCB1 inhibitor than the ABCB-reversing agent SDZ PSC833 (IC_{50} = 1.13 μM). The authors used porcine primary brain capillary endothelial cell monolayers as an *in vitro* system to study the effect of ABCB1 inhibition on the uptake of saquinavir into the cells. A 5.65-fold higher concentration of SDZ PSC833 was required to inhibit the ABCB1 mediated extrusion of saquinavir. The authors concluded that administering ritonavir with saquinavir may facilitate the brain uptake of the drug (Drewe et al., 1999).

Sudhakaran et al. investigated the effect of PSC833, a known ABCB1 inhibitor, and ritonavir, on the clearance index of indinavir (Sudhakaran et al., 2008). They carried

out the dual *in-vitro* perfusion of the isolated human placenta and found an increase in clearance index of indinavir between control (0.25 ± 0.03) and PSC833 treatment (0.37 ± 0.14). In contrast, clearance index of indinavir was observed to be unchanged between control (0.34 ± 0.14) and ritonavir treatment (0.39 ± 0.13) (Sudhakaran et al., 2008). The authors suggested the use of ABCB1 inhibitors to achieve higher transfer across the placenta for drugs which are substrates of ABCB1. However, since indinavir also interacts with ABCC1 (Srinivas et al., 1998) and ABCC2 (Huisman et al., 2002), it is possible that the above results may have been due to the contribution of other transporters.

Some protease inhibitors have also been reported to be inducers of ABCB1 expression. Vishnuvardhan et al. reported that acute treatment with lopinavir inhibits ABCB1 activity; however, on extended exposure lopinavir not only increased the efflux of rhodamine 123 but also the expression of ABCB1 protein and mRNA (Vishnuvardhan et al., 2003). The authors demonstrated that lopinavir potently inhibited ABCB1 mediated rhodamine 123 efflux in Caco-2 monolayer cells (IC_{50} = 1.7 μM). Upon chronic lopinavir exposure (72 hours) in LS180V cells, the content of intracellular rhodamine 123 was reduced by approximately 50%, which indicated increased efflux activity. In these cells, lopinavir induced ABCB1 mRNA and protein levels up to three-fold in a concentration dependent manner (Vishnuvardhan et al., 2003).

The influence of important anti-HIV drugs on ABCG2 activity *in vitro* has also been determined (Weiss et al., 2007). The authors assessed the ABCG2 inhibition by an increase in pheophorbide A accumulation in MDCKII-ABCG2 cells and compared it with the corresponding parental cell line MDCKII lacking human ABCG2. The authors

observed the following IC_{50} values regarding ABCG2 inhibition by the following anti-HIV drugs: lopinavir (7.66 μM) < nelfinavir (13.5 μM) < delavirdine (18.7 μM) ≤ efavirenz (20.6 μM) < saquinavir (27.4 μM) < atazanavir (69.1 μM) < amprenavir (181 μM) < abacavir (385 μM). Nevirapine and zidovudine were found to have a weak inhibitory effect. They could not estimate the inhibitory effects of ritonavir and tipranavir because of their low solubility. Other drugs in the study like indinavir, didanosine, emtricitabine, lamivudine, stavudine, tenofovir, and zalcitabine, were found not to have an inhibitory ABCG2 effect (Weiss et al., 2007).

Steady-state plasma concentrations in HIV-positive patients after administering the above drugs at their therapeutically used concentrations are reported in different studies. Mean steady-state peak ($C_{max,ss}$) and trough concentrations ($C_{min,ss}$) vary from 7.0±1.4 μM and 3.9±2.3 μM, respectively, for nelfinavir (dose = 1250 mg twice a day) to 35±20 μM and 15±10 μM, respectively, for delavirdine (dose = 400 mg thrice a day). $C_{max,ss}$ and $C_{min,ss}$ for lopinavir is reported as 15.6±5.9 μM and 11.3±4.6 μM (dose for lopinavir/ ritonavir as 400/100 mg twice daily); for ritonavir as 15.5±5.0 μM and 5.1±3.6 μM (dose administered as 600 mg every 12 hours); for atazanavir as 7.4±4.3 μM and 1.2±1.1 μM (dose for atazanavir/ ritonavir as 300/100 mg once daily); and for efavirenz (dose = 600 mg once daily) as 12.9 ± 3.7 μM and 5.6 ± 3.2 μM (www.efactsonline.com).

Gupta et al. investigated the effect of HIV protease inhibitors on ABCG2 efflux activity by measuring intracellular mitoxantrone fluorescence in human embryonic kidney (HEK) cells stably expressing wild-type ABCG2 (482R) and its two mutants (482T and 482G) (Gupta et al., 2004). Ritonavir, saquinavir, and nelfinavir were found to inhibit

wild-type ABCG2 (482R) with IC_{50} values of 19.5 ± 0.8 μM, 19.5 ± 7.6 μM, and 12.5 ± 4.1 μM, respectively. All the three drugs were observed to inhibit 482T and 482G with IC_{50} that was found to be twice that observed with 482R. Indinavir and amprenavir were found not to inhibit ABCG2 activity. In the same study, direct efflux of above mentioned radiolabelled drugs in HEK cells was studied. None of them were found to be substrates of ABCG2 (Gupta et al., 2004).

ABCC inhibition by indinavir, amprenavir, ritonavir, lamivudine or zidovudine has been investigated (Olson et al., 2002). Of the drugs studied, only ritonavir was found to inhibit the functional activity of ABCC1. The inhibitory effects were studied in UMCC-1/VP cells which over-express ABCC1 and the inhibitory activity was demonstrated by re-sensitization of ABCC1 over-expressing cells to cytotoxic effects of etoposide, a cytotoxic compound (Olson et al., 2002).

HIV protease inhibitors transfer poorly across the placenta. Cord/ maternal blood concentration ratios of protease inhibitors have been observed to range from 0.01 in case of indinavir to 0.27 in case of amprenavir (Marzolini et al., 2002; Chappuy et al., 2004b). In contrast, nucleoside and non-nucleoside reverse transcriptase inhibitors, which are much lesser plasma protein bound compared to protease inhibitors, transfer to a much greater extent. Cord/ maternal ratios range from 0.38 for didanosine to 1.32 for stavudine (Watts et al., 1991; Moodley et al., 1998; Mandelbrot et al., 2001; Marzolini et al., 2002; Chappuy et al., 2004a; Chappuy et al., 2004b). Similar results were observed in another study (Mirochnick et al., 2002). The authors in the above studies mention the lower placental transfer of protease inhibitors to be due to their efflux by ABCB1. However, the drugs are

known to be substrates of other ABC transporters as well. Therefore, other transporters in addition to ABCB1 may also contribute to the lower placental transfer observed with protease inhibitors. Also, the drugs' high plasma protein binding may also contribute, as discussed later in this review.

van Heeswijk et al. studied the pharmacokinetics of nelfinavir and its active metabolite hydroxy-t-butylamidenelfinavir (M8) during pregnancy and post partum in a group of patients (van Heeswijk et al., 2004). A 24% reduction in AUC_{0-12} of nelfinavir was observed during third trimester of pregnancy compared with post partum, though this reduction was not found to be statistically significant. The trough concentration (C_{12}) was also reduced by 57% during pregnancy. In addition, AUC of M8 was reduced by 70% in pregnant women. The authors found just 3 out of 11 patients with therapeutic nelfinavir trough concentrations during third trimester of pregnancy compared to 6 patients post partum. The authors recommended the adjustment of nelfinavir doses during late pregnancy to maintain therapeutic plasma concentrations. A decrease in exposure with other protease inhibitors in pregnant women has also been observed. For saquinavir (Acosta et al., 2001) and indinavir (Unadkat et al., 2007), values for $AUC_{0-8\ h}$ were 77% and 68% lower (respectively), antepartum compared to postpartum. Thus, dosage adjustments in pregnancy may be necessary for other protease inhibitors as well.

Mathias et al. evaluated whether systemic clearance, bioavailability, or plasma protein binding of nelfinavir was altered during pregnancy in mice (Mathias et al., 2006). The mice replicated the pregnancy related changes observed in the oral nelfinavir disposition in pregnant women. The authors observed a significant decrease in nelfinavir

C_{max} and AUC in pregnancy and a significant increase in oral plasma clearance of the drug, irrespective of whether or not it was normalized to body weight. The authors also determined if enhanced activity and/or expression of mouse CYP3A or ABCB1 or both were responsible for the reduced nelfinavir exposure observed during pregnancy. In contrast to oral administration, AUC and CL after intravenous administration were not significantly different in pregnant mice compared to those observed in non-pregnant mice. Bioavailability was significantly reduced in pregnant mice due to increased expression and activity of hepatic CYP3A. On the other hand, intestinal CYP3A as well as hepatic and intestinal ABCB1 expression were not found to be significantly different among pregnant and non-pregnant mice. Also, the percentage of nelfinavir unbound in plasma was found to be lower in pregnant mice compared to that in non-pregnant mice. This means that the increase in systemic clearance observed in pregnant mice was not due to a decrease in plasma protein binding. The authors concluded that the effect of pregnancy on nelfinavir disposition was due to a change in bioavailability and not due to change in plasma protein binding or systemic clearance of the drug.

Thus, it can be seen that HIV protease inhibitors interact with ABC transporters present in the placenta. This is important information to know at the time the physician tailors the patient's antiretroviral drug regimen. Besides ABC transporters, there are other transporters as well that are expressed in the placenta. ABC transporters being the major efflux transporters, there are other important uptake transporters like OATPs present in the placenta which may affect antiretroviral therapy outcomes.

EXPRESSION OF OATP -TRANSPORTERS IN THE PLACENTA

OATPs are plasma membrane transport proteins that modulate the uptake of a variety of compounds. Substrates of OATPs include bile salts, steroid conjugates, thyroid hormones, and several therapeutic drugs and other xenobiotics. They are expressed on the apical as well as basolateral membranes of polarized cells in liver, kidneys, intestine and blood brain barrier. They are encoded by genes of the solute carrier organic anion transporter (SLCO) superfamily, which includes 11 members in humans. The mechanism of substrate transport via OATPs is not completely understood. It is, however, believed to occur by electroneutral exchange, in which cellular uptake of organic anions is coupled to the efflux of neutralizing anions such as bicarbonate, glutathione or glutathione-S-conjugates (Kim, 2003; Mikkaichi et al., 2004; Niemi, 2007).

Of the known OATPs in humans, OATP1A2, OATP1B3, OATP4A1, OATP2B1, OATP3A1, and OATP1B1 are expressed in the placenta, as summarized in Table 2. Expression of OATPs in the human placenta has been investigated (Ugele et al., 2003). The authors found OATP2B1 (previously known as OATP-B) to be highly expressed in term placental tissue and freshly isolated mononucleated trophoblasts. OATP3A1 (OATP-D) and OATP4A1 (OATP-E) were highly and equally expressed in term placental tissue and in freshly isolated and cultured trophoblasts. Expression of OATP1A2 (OATP-A) was low in term placental tissue and freshly isolated mononucleated trophoblasts compared to OATP1B1 (OATP-C) whose expression was low in term placental tissue but was not detectable in isolated and cultured trophoblasts. OATP1B3 (OATP-8) was intermediately expressed in the placental tissue (Ugele et al., 2003).

Table 2: Summary of OATP transporters expressed in the placenta

Transporter	**Expression in:** **i) term placental tissue** **ii) freshly isolated mononucleated trophoblasts (Ugele et al., 2003)**	**Localization**	**Expression in the third trimester compared to first trimester (Patel et al., 2003)**
OATP1A2 (OATP-A)	Low in both (i) and (ii)		down regulated 8-fold
OATP1B3 (OATP-8)	Intermediately expressed in (i)		
OATP4A1 (OATP-E)	Highly and equally expressed in (i) and (ii)	Apical (Sato et al., 2003)	Not differentially expressed in the two trimesters
OATP2B1 (OATP-B)	High in both (i) and (ii)	Basolateral, throughout the gestation (St-Pierre et al., 2002)	
OATP3A1 (OATP-D)	Highly and equally expressed in (i) and (ii)		down regulated 17-fold
OATP1B1 (OATP-C)	Low in (i); not detectable in (ii)		Not differentially expressed in the two trimesters

St. Pierre et al. showed by Western blotting and immunohistochemistry that OATP2B1 was expressed at the basolateral surface of the syncytiotrophoblast throughout gestation and was also localized in cytotrophoblasts before differentiation to the syncytiotrophoblast (St-Pierre et al., 2002). Western blotting analysis carried out by Sato et al. showed a single band for OATP4A1 in the human placenta. In addition, immunohistochemsitry revealed that it was predominantly expressed at the apical surface of the syncytiotrophoblasts in the placenta, suggesting a functional role for the transplacental transfer of thyroid hormone (Sato et al., 2003). Expression of OATP4A1 in the placenta is also reported elsewhere, where the authors suggested its importance in maternal thyroid hormone transport to the developing fetus (Hagenbuch, 2007).

Semi-quantitative expression analysis of OATP1A2, OATP1B1, OATP3A1, and OATP4A1, along with a few other genes was carried out by Patel et al. in first and third trimester human placenta (Patel et al., 2003). Expression of OATP1A2 and OATP3A1 was down regulated eight and seventeen fold, respectively, in the third trimester compared to the first trimester. In contrast, OATP1B1 and OATP4A1 were not differentially expressed in first and third trimester placentae. OATP1B1 gene expression was not detected in the third trimester placenta, while low levels of transcripts were detected in the first trimester placentae (Patel et al., 2003). Serrano et al. used cytokeratin-7-positive trophoblast cells (hTr) isolated from human term placentas and the choriocarcinoma cell lines (hCC) BeWo, Jeg-3 and JAr to investigate the expression of OATP2B1, OATP1B3, OATP1A2, and OATP1B1, along with other genes (Serrano et al., 2007). In hTr, the expression was high for OATP2B1 and OATP1B3, very low for OATP1A2 and not detectable for OATP1B1.

Expression patterns in hCC mimicked those in hTr, although the authors did find some important cell line-specific differences (Serrano et al., 2007).

Briz et al. investigated the role of OATPs in the transport of unconjugated bilirubin by the placenta-maternal liver tandem (Briz et al., 2003). The authors obtained RNA from human liver (hL), human placenta (hPl) at term, and purified cytokeratin-7-positive mononucleated human trophoblast cells (hTCs). Using analytical RT-PCR, agarose gel electrophoresis separation and sequencing, mRNA of OATP1A2 and OATP1B3 was identified in hL, hPl, and hTCs where as that of OATP1B1 was only detectable in hL. Real-time quantitative RT-PCR revealed that in hL the abundance of mRNA was OATP1B3 > OATP1B1 >> OATP1A2, where as in hPl and hTCs, it was OATP1B3 >> OATP1A2 >> OATP1B1. The authors observed the expression levels for these OATPs to follow the rank order hL >> hTCs > hPl (Briz et al., 2003).

HIV PROTEASE INHIBITORS AND OATP TRANSPORTERS

As mentioned earlier, OATPs may have an important role in human drug disposition; their presence on the apical and/or basolateral side of the placental syncytiotrophoblast may affect the extent to which HIV protease inhibitors get transferred across the placenta. HIV protease inhibitors have been shown to be inhibitors of OATP2B1 in Caco-2 cells (Kis et al., 2008). RT-PCR and immunoblotting was used to determine the OATP2B1 mRNA and protein expression in OATP2B1-overexpressing MDCKII and Caco-2 cells. The authors screened a number of antiretroviral compounds for inhibition of OATP2B1-mediated transport by measuring estrone-3-sulfate in MDCKII/OATP2B1 and

Caco-2 cells in the presence or absence of each drug. They also determined the IC_{50} values by performing concentration-dependent inhibition studies. Atazanavir and ritonavir were not found to be transported by OATP2B1, along with several other protease inhibitors; however, they were potent inhibitors in both cell models. In MDCKII/OATP2B1 cells, the IC_{50} values were 3.9 μM for atazanavir, 2.2 μM for ritonavir, 0.72 μM for lopinavir, 0.67μM for nelfinavir, and 0.88 μM for tipranavir. The corresponding values in Caco-2 cells were 2.5 μM, 1.1 μM, 1.7 μM, 1.8 μM, and 0.72μM. The authors did not observe any significant inhibition for other classes of antiretrovirals and some protease inhibitors at clinically relevant concentrations (Kis et al., 2008).

In other studies, human OATPs, ABCC, ABCB1 and lipophilicity were important determinants of the extent of cellular uptake and retention of saquinavir and lopinavir in the used T-cell lines (CEM, CEM_{VBL}, and CEM_{E1000}) and in peripheral blood mononuclear cells (Janneh et al., 2008). MK571, furosemide (inhibitors of ABCC) and XR9576 (inhibitor of ABCB1) increased the uptake of both the drugs whereas estrone-3-sulfate (hOATP substrate) and montelukast (hOATP inhibitor) reduced the uptake in some but not all of the cells (Janneh et al., 2008). Additionally, lopinavir is a substrate of OATP1B1 (Shallcross et al., 2008a) and OATP1A2 (Shallcross et al., 2008b).

In a study carried out with saquinavir, *Xenopus oocytes* injected with OATP1B1 cRNA demonstrated an approximately 50% increase in the uptake of ^{14}C-saquinavir compared to water injected oocytes after 1 hour, and a 2-fold increase after 2 hours (Kutscher et al., 2005). In addition, a linear increase in uptake of ^{14}C-saquinavir with time was at a significantly greater rate in OATP1B1 injected oocytes compared to water

injected oocytes. This suggested an involvement of OATP1B1 in saquinavir uptake (Kutscher et al., 2005).

Thus, there have been studies that report interaction of protease inhibitors with OATPs. With protease inhibitors being substrates of ABC as well as OATP transporters, there may be a possibility of interplay between these classes of transporters which would affect the extent of drug getting transferred across the placenta. Expression of these transporters in the experimental model used and in the individual patients in the clinical situation would also play an important role.

PLASMA PROTEIN BINDING ISSUES

The placenta separates maternal and fetal blood circulations, wherein, the concentrations of drug binding proteins are high in both circulations. However, they are unequal and dynamic over time. This protein concentration gradient favors partitioning of total drug on the maternal side for highly plasma protein bound drugs like HIV protease inhibitors (most > 98%). It has been shown that concentrations of plasma proteins such as α_1-acid glycoprotein and albumin change during the gestation period. Between 12 and 41 weeks gestation, maternal serum albumin concentration ranges from 311 μM (20.5 g/L) to 583 μM (38.5 g/L), while fetal serum albumin ranges from 114 μM (7.5 g/L) to 603 μM (39.8 g/L) (Krauer et al., 1984), and as high as 659 μM (43.5 g/L) (Nation, 1981). Maternal serum AAG concentration, on the other hand, ranges from 7 μM (0.29 g/L) to 24 μM (1.05 g/L) (Krauer et al., 1984) and as high as 46 μM (2 g/L) in the presence of acute or chronic inflammation (Chu et al., 1981). Fetal serum AAG concentrations range from ≤ 0.2 μM

(0.01 g/L) to 9 μM (0.41 g/L) (Krauer et al., 1984). Similar plasma/serum protein concentration ranges have been observed in other studies as well (Laurell, 1968; Ganrot, 1972; Chu et al., 1981; Nation, 1981; Wood and Wood, 1981; Raynes, 1982; Denson et al., 1984; Krauer et al., 1984). Thus, the binding of highly plasma protein bound drugs such as HIV protease inhibitors, changes during gestation.

Additionally, because of concentration differences of proteins in the maternal and fetal circulations, it is important to determine the unbound fraction of the protease inhibitors, not only in the maternal blood but also the fetal/cord blood. Sudhakaran et al observed a higher unbound fraction of indinavir and saquinavir in cord plasma compared to maternal plasma and suggested this binding differential to be due to the transplacental AAG concentration gradient (Sudhakaran et al., 2006). Assuming that the clearance would be same in the maternal and fetal compartment, the protein concentration gradient that exists in the maternal and fetal circulations during pregnancy may unfavorably affect partitioning of the total drug into the fetal compartment, while free drug concentrations may be equivalent.

INTRACELLULAR ACCUMULATION OF HIV PROTEASE INHIBITORS

Intracellular accumulation of HIV protease inhibitors has been investigated and marked differences have been observed among them *in vitro*. Jones et al. used CEM cells to study this and found a hierarchy in the intracellular accumulation of four protease inhibitors they studied in the rank order nelfinavir > saquinavir > ritonavir > indinavir (Jones et al., 2001b). They also used CEM cells treated with vinblastine (CEM_{VBL}) or

epirubicin (CEM_{E1000}) that had an increased expression of ABCB1 or ABCC1, respectively, to study the effect of active transport on this accumulation. A significant reduction in the intracellular accumulation of nelfinavir, saquinavir, and ritonavir in CEM_{VBL} cells and of saquinavir and ritonavir in CEM_{E1000} cells was observed. The effect of inhibition of active transport was also studied by incubating the cells at 4°C and in the presence of 2-deoxyglucose and rotenone. This resulted in a reduction in accumulation of saquinavir and ritonavir; however, it had no effect on indinavir accumulation in all cell types. For nelfinavir, an increase in accumulation was observed in CEM_{VBL} cells as a result of inhibition of active transport. The authors concluded that ABCB1 and ABCC1 along with some active influx mechanisms may contribute to the drug accumulation of protease inhibitors (Jones et al., 2001b). Meaden et al. investigated whether differences in expression of ABCB1 and ABCC1 in human lymphocytes correlated with intracellular concentrations of ritonavir and saquinavir (Meaden et al., 2002). The authors determined the expression of these transporters in peripheral blood mononuclear cells isolated from HIV positive patients and also measured the plasma and intracellular ritonavir and saquinavir concentrations. An increased expression of these transporters was associated with lower intracellular accumulation of the drugs (Meaden et al., 2002).

Khoo et al. determined the intracellular accumulation of saquinavir, ritonavir, and indinavir in 50 HIV-positive patients (Khoo et al., 2002). The authors collected paired plasma and intracellular samples (samples from peripheral blood mononuclear cells) over a full dosing interval from patients and expressed the data as intracellular/ plasma drug concentration ratios. Similar to previously published results, the authors observed a

hierarchy of intracellular accumulation in the rank order saquinavir > ritonavir > indinavir (Khoo et al., 2002). The authors also performed the fractionation of U937 cells (human leukemic monocyte lymphoma cells) and studied the intracellular localization of the above drugs. Efflux kinetics of saquinavir with time and at different temperatures (37°C and 4°C) was also studied. It was suggested by the authors that the recovery of protease inhibitors associated with the cell pellets represented the drug that was present within the cell compartments, such as the cytoplasm and mitochondria, rather than that bound to the cell surface membrane. The radiolabelled compounds were detected in all sub-cellular fractions (cytosolic, nuclear, heavy and light mitochondrial). Though the authors did not discuss this, it seems that the PIs accumulated to a maximum extent in the mitochondrial fractions compared to the nuclear or cytosolic fractions. The authors also found the efflux out of the cells to be time and temperature dependent (Khoo et al., 2002).

Mechanisms involved in controlling the intracellular penetration of HIV protease inhibitors have been reviewed earlier (Hoggard and Owen, 2003; Ford et al., 2004). The authors in these reviews discuss that the plasma drug concentrations of these compounds depend on the extensive first pass metabolism which contributes to low and variable bioavailability. These drugs are also substrates of several transmembrane uptake (OATP) and efflux (ABC) transporters (Hoggard and Owen, 2003; Ford et al., 2004).

Most of the protease inhibitors are highly plasma protein bound (>95%, except indinavir which is 60% bound) and this reduces the fraction of drug available for cell penetration or crossing the placenta. These drugs predominantly bind to AAG, which has a number of genetic variants and shows variable expression in HIV patients (Boffito et al.,

2002). Reduced intracellular protease inhibitor concentrations (saquinavir- 31.5 μM to 7.4 μM, ritonavir- 8.8 μM to 1.6 μM, and indinavir- 3.0 μM to 1.5 μM) have been observed in the presence of 2.0 mg/ml AAG (Jones et al., 2001a). However, since protease inhibitors are low extraction ratio drugs, a change in unbound fraction of these drugs may not be clinically relevant but there will be a change in total drug concentration as well. Determination of both total and unbound plasma drug concentrations is, therefore, necessary to get a better insight into the drug available for pharmacological effect.

As the protease inhibitors are highly bound to extracellular proteins, they may also be highly bound to intracellular proteins; there are many protein rich areas within the cell as well (microfilaments, microtubules, proteins embedded in cellular, nuclear and mitochondrial membranes, and phospholipids and proteins in the endoplasmic reticulum). The drugs show differential accumulation; mechanisms suggested as mediating these intracellular accumulation differences include differences in lipophilicity, intracellular protein binding, and ion trapping. These drugs are also substrates of efflux and uptake transporters, which may contribute to differences in intracellular accumulation (Hoggard and Owen, 2003; Ford et al., 2004).

WHY LOPINAVIR/ RITONAVIR?

As mentioned earlier, the use of HAART regimens has significantly reduced the perinatal transmission to less than 2% (Watts, 2006). It has also been discussed earlier that a lower placental transfer of protease inhibitors compared to other antiretrovirals in a HAART regimen may result in viral resistance leading to treatment failure.

As recently as two years ago, nelfinavir was used as a first line agent in the treatment of pregnant HIV infected patients. This was because it was the protease inhibitor with the most pharmacokinetic data and clinical experience with use in pregnancy (Watts, 2006). In 2007, however, the FDA recommended not to offer regimens containing Viracept (nelfinavir mesylate) to pregnant women who needed to begin an antiretroviral therapy. As a precaution, they also recommended pregnant women receiving Viracept to be switched to a therapy consisting of an alternative protease inhibitor. This was because excess levels of ethyl methanesulfonate (EMS) were detected in Roche Ltd- manufactured active pharmaceutical ingredient of Viracept. Because data from animal studies were available that indicated EMS as teratogenic, mutagenic, and a potential human carcinogen; FDA recommended pregnant women to limit their exposure to EMS during pregnancy. Maintaining the health of the mother and the prevention of viral transmission to the fetus were goals of paramount importance (http://www.fda.gov/MedWatch/safety/2007/VIRACEPT_HCPLetter_9_10_07.pdf).

Lopinavir/ritonavir is approved for use in combination antiretroviral therapy for HIV infection in adults and children (http://www.rxabbott.com/pdf/kaletratabpi.pdf). Ritonavir is used in the combination as a boosting agent that inhibits the CYP3A4 mediated metabolism of lopinavir providing increased plasma levels of lopinavir. Lopinavir/ritonavir is assigned Food and Drug Administration (FDA) Pregnancy Category C, indicating that risk to pregnancy cannot be ruled out; adequately powered human studies are lacking; and animal studies are either positive for fetal risk or unavailable (http://www.rxabbott.com/pdf/kaletratabpi.pdf,http://www.fda.gov/cder/pediatric/21cfr201

57.htm). In animal studies of lopinavir/ ritonavir, no treatment related malformations were reported, however, embryonic developmental toxicities occurred in rats receiving maternally toxic doses (http://www.rxabbott.com/pdf/kaletratabpi.pdf). A recent study used the Antiretroviral Pregnancy Registry data to estimate the birth defect risk after pregnancy exposures to lopinavir/ritonavir (Roberts et al., 2009). The authors did not observe a significant difference between the prevalence of birth defects among infants prenatally exposed to lopinavir/ritonavir and the internal (first trimester exposures compared with combined second/third trimester lopinavir/ ritonavir exposures) or external (Metropolitan Atlanta Congenital Defects Program) comparison groups. The data obtained in this study has reassured patients and clinicians about the safety of lopinavir/ritonavir in the treatment of HIV-positive pregnant women (Roberts et al., 2009). This explains why lopinavir/ ritonavir is currently the preferred protease inhibitor recommended by the US Department of Health and Human Services for use with other antiretrovirals during pregnancy (http://aidsinfo.nih.gov/ContentFiles/PerinatalGL.pdf). As a result, the studies presented herein focus on lopinavir.

WHY BeWo CELL LINE AS A MODEL?

The extent of fetal exposure to a drug can be best studied by carrying out clinical studies with pregnant women; however, due to ethical and practical issues concerning maternal and fetal health this approach is not used. Also, because of structural and physiologic differences between human placenta and placenta of other species, extrapolation of data from animal models to humans may not give the actual picture of the

mechanisms involved. Several *ex vivo* and *in vitro* models are used to study placental transfer of compounds, which include perfused placental model, trophoblast tissue preparations (villous preparations, and microvillous brush-border membrane and basal membrane vesicles), and trophoblast cultures. These techniques have been reviewed well in literature (Mitra and Audus, 2008).

The BeWo cell line is a choriocarcinoma derived cell line that forms polarized, confluent monolayers. It is of human origin, is easy to maintain and grows to a confluent monolayer in 4-5 days. The cells are polarized with respect to expression of transporters on the apical and basolateral membranes and of the enzymes as well. Several ABC and OATP transporters are expressed in BeWo cells, as previously discussed. Transport studies can be carried out using either transwell inserts or side-by-side diffusion chambers (Mitra and Audus, 2008). Also, the BeWo cell line appeared to be a good model of the human syncytiotrophoblast and as a result, we chose this cell culture model of the human placental trophoblast for our studies.

CHAPTER 2 SIGNIFICANCE, HYPOTHESIS AND SPECIFIC AIMS

HIV is a deadly virus that has infected close to 60 million people since its onset, with almost 20 million dying from its complications. Of the more than 40 million people with HIV infection today, close to half of them are women, and more than 3 million are children under the age of 15 (Libman and Makadon, 2007). Over the past decade, HAART has led to a significant increase in life expectancy. In pregnant HIV infected patients, HIV may get transmitted from the mother to the fetus. The maximum chance of transmission of the virus is at the time of birth through the mucous membranes. HAART serves two goals in HIV-infected pregnant patients; providing adequate treatment for the mother and preventing viral transmission to the fetus. The use of HAART regimens has led to a significant reduction in occurrence of perinatal transmission to less than 2% (Watts, 2006). Among the antiretroviral drugs used, there are differences in the extent of transfer of these drugs across the placenta; HIV protease inhibitors are particularly poorly transferred.

The placenta separates maternal and fetal blood circulations, wherein, the concentrations of drug binding proteins are high in both circulations. However, they are unequal and dynamic over time. This protein concentration gradient favors partitioning of

total drug on the maternal side for highly plasma protein bound drugs like HIV protease inhibitors (most > 98 %). It has been shown that concentrations of plasma proteins such as α_1-acid glycoprotein and albumin change during the gestation period (Krauer et al., 1984). Thus, the binding of highly plasma protein bound drugs such as HIV protease inhibitors, changes during gestation. Additionally, because of concentration differences of proteins in the maternal and fetal circulations, it is important to determine the unbound fraction of the protease inhibitors, not only in the maternal blood but also the fetal/cord blood.

One of the major reasons responsible for poor placental transfer of HIV protease inhibitors is that they are substrates of ABC transporters present on the maternal side of the placental syncytiotrophoblast. These transporters efflux the drugs back into the maternal circulation, which may result in failure to prevent vertical transmission. Inhibition of these transporters will result in an increase in placental transfer of protease inhibitors. Sudhakaran et al. have suggested the use of ABCB1 inhibitors to achieve higher transfer across the placenta for drugs which are substrates of ABCB1 (Sudhakaran et al., 2008). Among HIV protease inhibitors, saquinavir is the most studied drug. For this study, however, lopinavir was chosen as the drug to study as its combination with ritonavir is currently the drug combination of choice to treat HIV infected pregnant women. Limited data are available for lopinavir. Additionally, protease inhibitors also interact with ABCC1 (Srinivas et al., 1998) and ABCC2 (Huisman et al., 2002). Therefore, ABCC1 and ABCC2, which have not been studied extensively with protease inhibitors, may also be involved in the low lopinavir placental transfer.

Among the experimental models available to study transplacental transfer of drugs, BeWo cell line is a cell line that is of human placental origin, is easy to grow, the cells reach confluence within 4-5 days, and expresses several transporters and enzymes like the human placenta. In addition, this cell line has not been used extensively to study transplacental transfer of HIV protease inhibitors.

HYPOTHESIS AND SPECIFIC AIMS:

Inhibiting the placental ABC transporters increases the transfer of HIV protease inhibitors in the maternal-to-fetal direction.

As a result, the purpose of this study was:

1. To determine the unbound fraction of lopinavir in cord blood.
2. To characterize the kinetics of lopinavir binding to human AAG and HSA and the effects of ritonavir.
3. To characterize lopinavir uptake and efflux in the BeWo cell model.

CHAPTER 3 MATERIALS AND METHODS

PROTEIN BINDING STUDIES:

AAG was obtained from Fisher Scientific (catalog number ICN15390510) and from Sigma Aldrich (catalog numbers G9885-10MG and G9885-25MG). HSA was obtained from Fisher Scientific (catalog number ICN823234). Lopinavir and ^{3}H-lopinavir (specific activity of 100-139 mCi/mmol) were obtained from AK Scientific, CA (catalog number 69314) and Moravek Biochemicals, CA (catalog number MT 1648), respectively. Ritonavir was obtained from Sigma Aldrich (catalog number R535000) and Bosche Scientific, NJ (catalog number R1692).

Purity of ^{3}H-lopinavir was tested using HPLC. The analysis was done using an Alltima C18 column with UV detection at 254 nm. The mobile phase consisted of 100% acetonitrile-1% acetic acid in water (55:45 v/v). 50 µL of a 0.01 µCi/µL solution of ^{3}H-lopinavir in mobile phase was injected into the system. A single peak of lopinavir was detected at a retention time of 4.3 minutes and the purity of ^{3}H-lopinavir was 96.1%.

Placentae were obtained from 4 women (clinical data of the patients shown in Table 3) from cesarean section deliveries following normal pregnancies at the VCU Medical Center Hospital. The study was approved by the VCU Institutional Review Board (protocol #4212 – Appendix I) and informed consent was obtained from patients prior to

delivery. Patients with hypertension, diabetes, preeclampsia, HIV infection or febrile illness; history of smoking, alcohol or drug abuse were excluded. Maternal blood samples were not available in this study. Cord blood was obtained from placentae within 30 minutes of birth, allowed to clot on ice, and centrifuged to obtain serum. Maternal serum samples were purchased from BioChemed Services (Winchester, VA). These samples were obtained from non-smoking pregnant patients aged 19-34 years between 17 and 27 weeks gestation (Table 4). Body mass index (BMI) was calculated for each of these pregnant patients. The website used to calculate BMI is http://www.nhlbisupport.com/bmi/.

Rapid equilibrium dialysis (RED, Figure 1; Pierce No. 89809), with a molecular weight cut-off of 8,000 Daltons, was used to determine the protein binding of lopinavir in maternal serum and in serum obtained from cord blood. These protein binding experiments with serum samples were followed by experiments with varying concentrations of lopinavir, AAG and HSA. The protein solutions were prepared in phosphate buffered saline, pH 7.4 (PBS). The final concentrations of lopinavir were 0.1, 0.32, 1, 3, 10, 30, and 100 μM. Protein binding of lopinavir was determined in the presence of 7 μM (0.3 g/L) and 23 μM (1.0 g/L) AAG, and 15 μM (1 g/L), 152 μM (10 g/L), and 758 μM (50 g/L) HSA.

Table 3: Clinical data for patients undergoing C-section delivery following normal pregnancy

Patient	Age (years)	Gestational Age (Weeks/Days)	Race	Placental Weight (g)	Cord serum experiments	Tissue binding experiments
4212-034	36	39/1	Hispanic	786		X
4212-036	23	39/2	African American	839	X	
4212-037	23	39/0	White	822	X	
4212-038	45	38/3	African American	567	X	X
4212-040	25	39/0	African American	868	X	X

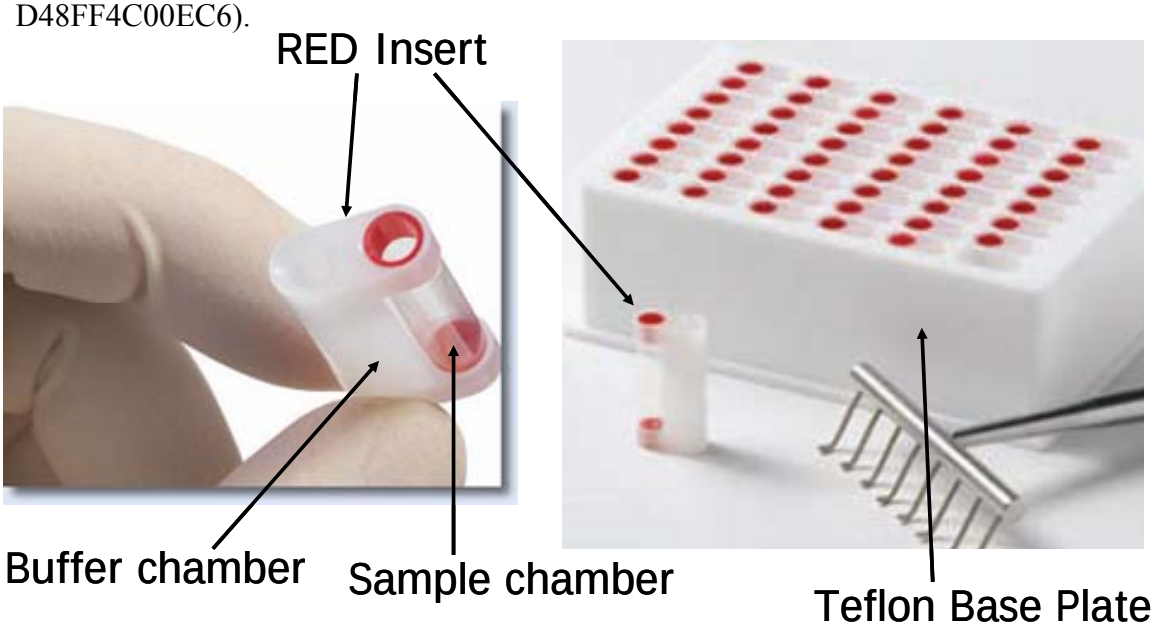
The protein solution (500 µl) or maternal serum (200 µl) containing the drug was added to the sample (inner) chamber and 750 µl of PBS (or 350 µl for maternal serum experiments) was added in the buffer (outer) chamber. Non-specific binding was determined by adding PBS in both the compartments with the solution in the sample chamber containing ^{3}H-lopinavir alone. The Teflon base plate containing the inserts was covered and incubated at 37ºC on an orbital shaker at 100 rpm. Samples (50 µl for serum experiments and 100 µl for the other experiments) were removed from each side at various times up to 16 hours, and ^{3}H-lopinavir in sample and buffer compartments was detected by liquid scintillation counting on a Packard TR2800 scintillation analyzer (Perkin Elmer). Fraction unbound was determined by taking the ratio of mass of radioactivity observed in the buffer chamber to that in the sample chamber. All determinations were made in triplicates.

The equation for drug binding to a protein is given by:

$$\frac{B}{P_T} = \frac{N[Lopinavir]_f}{K_D + [Lopinavir]_f} \qquad \text{Equation (1)}$$

where, B is the bound drug concentration, P_T is the total protein concentration, N is the number of binding sites per protein molecule, K_D is the equilibrium dissociation constant and $[Lopinavir]_f$ is the free drug concentration. Binding parameters N and K_D were determined by non-linear least-squares regression using equation 1 (GraphPad Prism version 5).

Figure 1: Rapid Equilibrium Dialysis (modified version; original source: http://www.piercenet.com/Products/Browse.cfm?fldID=8C6CA217-75C3-4019-AE9C-D48FF4C00EC6).



These studies were followed by selecting a physiologic maternal concentration of AAG (9 μM = 0.38 g/L) or HSA (530 μM = 35 g/L), and carrying out the determination of lopinavir protein binding at lopinavir concentrations ranging from 0.1 to 30 μM in the presence of different concentrations (0, 1, 10, 25, and 50 μM) of ritonavir. Lopinavir protein binding to HSA (530 μM = 35 g/L) was also determined in the presence of a higher ritonavir concentration of 100 μM. Data from HSA and AAG experiments were analyzed by two-way ANOVA with Bonferroni's post-test; significance was assessed at $p<0.05$. Binding of lopinavir in cord and maternal serum was compared using an unpaired t-test with Welch's correction (GraphPad Prism version 5).

BeWo CELL CULTURE STUDIES:

The BeWo cell line is originally derived from a human choriocarcinoma. The BeWo cell line (Schwartz clone; passage 30) was obtained from Dr. Kenneth L. Audus (The University of Kansas Medical Center, Kansas City, KS). BeWo cells in these studies were used between passages **36-64**.

Cell culture medium:

This was prepared using Dulbecco's modified Eagle's medium (DMEM) (high glucose with 4500 mg/L glucose and L-glutamine, without sodium bicarbonate, powder, cell culture tested; Sigma Aldrich, catalog number D5648-10X1L), fetal bovine serum (FBS) (heat inactivated, Quality Biological Inc., catalog number QBI110001101HI), L-glutamine (200 mM solution; Fisher, catalog number MT25005CIRF), penicillin/

streptomycin (10,000 U/mL; Invitrogen, catalog number 15140122, Quality Biological Inc., catalog number QBI120095061) and MEM nonessential amino acids (100x solution; Fisher, catalog number MT25025CIRF).

To prepare the media, protocol described previously (Bode et al., 2006) was followed and the steps are explained in brief in Appendix II.

Culturing of BeWo cells:

10X PBS, pH 7.4 was obtained from Quality Biological Inc. (catalog number QBI119069101). 10X trypsin and trypan blue were obtained from Invitrogen (catalog number 15400054) and Sigma Aldrich (catalog number T8154-100mL), respectively.

BeWo cells were grown and cultured according to the protocol previously described (Bode et al., 2006), except for a few minor modifications, which are described in Appendix II.

Growth of BeWo cells on standard cell culture plates:

1. The cell pellet was obtained from trypsinization of a 150 cm^2 flask.
2. The supernatant media was aspirated and the cell pellet was resuspended in 10 mL of the medium.
3. After seeding part of this cell suspension to one or more 150 cm^2 flasks, additional medium was added to the remaining cell suspension so as to come up with a total volume of cell suspension enough for the required number of 12 well plates. 1 mL

of the cell suspension was seeded to each well of a 12-well plate, which was shook to evenly distribute the cells.

4. The cells were fed every other day subsequently and the uptake/efflux experiments carried out when the cells were at least 90-95% confluent, which usually occurred 4-5 days after plating.

Efflux experiments:

Preliminary time course experiments:

After the BeWo cells grown on standard 12-well plates reached 90-95% confluence, efflux experiments were carried out to determine the efflux of ^{3}H-lopinavir from BeWo cells in the presence and absence of different inhibitors of ABC transporters present on the maternal side of the placental syncytiotrophoblast. The media was aspirated and the cells were washed once with Dulbecco's Phosphate Buffered Saline (DPBS). The cells were then incubated for 30 minutes with DPBS. The buffer was aspirated after 30 minutes and replaced by the uptake buffer which consisted of ^{3}H-lopinavir (0.32 μM) in DPBS ± any inhibitors of ABC transporters. Verapamil, a known ABCB1 inhibitor, or sodium orthovanadate, an inhibitor known to inhibit all ATP dependent processes, were used at concentrations of 100 μM or 500 μM, respectively. There was a one hour pre-incubation period with 1 mM sodium orthovanadate for the experiment with sodium orthovanadate. The inhibitors were added at the uptake step so that the cells are equilibrated with the inhibitor before the efflux step. In the later experiments, however, the inhibitors were not included at the uptake step. The cells were incubated with the uptake

buffer for 5 minutes. The entire buffer solution was aspirated after 5 minutes and the cells were then incubated with the efflux buffer which comprised of inhibitor solutions in DPBS for various time intervals (0 min, 2 min, 5 min, 10 min, or 20 min). The entire buffer solutions were then collected and the samples were analyzed by mixing 5 mL of scintillation cocktail and counting on a Liquid Scintillation Analyzer (Tri-Carb 2800TR, Perkin Elmer). To each well of the 12-well plate, 1 mL of lysis buffer (0.5% Triton X-100 in 0.2 N NaOH) was then added. The plate was covered and incubated in the incubator at 37°C for 2 hours, or at 4°C overnight. After the incubation period, entire contents of each well (~ 1-1.2 mL) were mixed by pipetting and collected in different scintillation vials. Scintillation cocktail (5mL) was then added to the samples which were then analyzed by liquid scintillation counting.

An efflux experiment was also done in the presence or absence of verapamil (100μM) without any cells on the plate. This was done to determine any non-specific binding of ^{3}H-lopinavir to the plastic.

An efflux experiment was also done in the presence or absence of GF120918 (1μM, known ABCB1 inhibitor) and ritonavir (10 μM). Ritonavir was used since its combination with lopinavir is used clinically to treat HIV-infected pregnant patients.

Efflux experiments at 37°C and 4°C:

Efflux experiments with ^{3}H-lopinavir (0.32 μM) and verapamil (100 μM) were also performed at different temperatures to differentiate between the uptake and binding components of lopinavir transport across the BeWo cells. The only difference between

these and earlier timed experiments was that these experiments were done by incubating the cells at different temperatures (37°C and 4°C) for 5 or 10 minutes in the uptake step followed by a 20 minute efflux step.

Obtaining cell pellets for cell pellet binding experiments:

1. The cell pellet was obtained from trypsinization of a 150 cm^2 flask.
2. The supernatant media was aspirated and the cell pellet was resuspended in 10 mL of the medium.
3. After seeding part of this cell suspension to one or more 150 cm^2 flasks, the remaining cell suspension was centrifuged again to pellet at 300 G for 8 minutes at room temperature.
4. The cell pellet was uniformly resuspended in 1 mL of medium and the entire cell suspension was transferred to a pre-labeled eppendorf tube. The cells in the tube were then stored at -80°C for cell pellet binding experiments.

BeWo cell and placental tissue homogenate binding experiments with ^{3}H-lopinavir:

^{3}H-lopinavir binding to the placental tissue was determined using the RED device. Approximately 500 mg of placental tissue (patient numbers 4212-034, -038, and -040) frozen previously at -80°C was homogenized in PBS (3 mL). 500 µL of this homogenized tissue from each patient containing ^{3}H-lopinavir (0.64 µL of 0.025 µCi/µL) was transferred in triplicate to the sample (inner) chamber of the RED inserts. PBS (750µL) was added to the buffer (outer) chamber and the Teflon base plate containing the inserts was covered and

incubated at 37°C on an orbital shaker at 100 rpm. Samples (100 μL) were collected at the end of 16 hours, and ^{3}H-lopinavir in sample and buffer compartments was detected by liquid scintillation counting. Fraction unbound was determined as previously. The remaining placental tissue homogenate was used to determine the protein content using the Lowry protein assay (Appendix III).

A similar experiment was also carried out with BeWo cell pellets stored at -80°C (cell pellets from passage numbers 34, 39, 40, 41, 43, and 45). The cell suspensions in DMEM stored at -80°C were centrifuged at 300 G (approx. 2000 rpms) for 8 minutes at 4°C using Microfuge 18 Centrifuge (Beckman Coulter). The pellets obtained were resuspended in DPBS without Ca/Mg (4°C) and centrifuged again at 2000 rpms for 8 min at 4°C. This was done twice before resuspending the pellets in 1 mL PBS (4°C). The cell suspensions obtained from the above step were then pooled together and transferred to a 14mL Falcon 2059 tube. EDTA was added to the cell suspension (to provide 1 mM EDTA) along with protease inhibitor cocktail (240 μL of 1 tablet/ 2 mL solution in water). This pooled cell suspension was homogenized on ice and was used to carry out the cell homogenate binding experiments at 37°C and 4°C in the presence or absence of verapamil (100 μM) or ritonavir (10 μM). The remaining suspension was also used to determine the protein content using the Lowry protein assay (Appendix III).

Uptake experiments:

Preliminary time course experiments:

After the BeWo cells grown on standard 12-well plates reached 90-95% confluence, uptake experiments were carried out to determine the uptake of ^{3}H-lopinavir in BeWo cells in the presence and absence of different inhibitors of ABC transporters present on the maternal side of the placental syncytiotrophoblast. These experiments were carried out in a similar way, until the addition of the uptake buffers, as the preliminary time course efflux experiments. The cells were incubated with the uptake buffer for various time intervals (0 min, 2 min, 5 min, 10 min, or 20 min). The entire buffer solutions were collected and the samples were analyzed by mixing 5 mL of scintillation cocktail and counting on a Liquid Scintillation Analyzer (Tri-Carb 2800TR, Perkin Elmer). To each well of the 12-well plate, 1 mL of lysis buffer comprising of 0.5% Triton X-100 in 0.2 N NaOH was added. The plate was covered and incubated in the incubator at 37°C for 2 hours, or at 4°C overnight. After the incubation period, the contents of each well were mixed by pipetting and collected in different scintillation vials. Scintillation cocktail (5mL) was added to the samples which were then analyzed by liquid scintillation counting.

Uptake experiments at 37°C and 4°C:

Uptake experiments with ^{3}H-lopinavir (0.32 μM) and verapamil (100 μM) were also performed at different temperatures to differentiate between the uptake and binding components of lopinavir transport across the BeWo cells. The only difference between

these and earlier timed experiments was that these experiments were done by incubating the cells at different temperatures (37°C and 4°C) for 10 minutes.

An uptake experiment at 37°C was also done in the presence of bromosulfophthalein (BSP, 50 μM) and taurocholate (TC, 100 μM) as inhibitors of certain OATP transporters. *Trans*-stimulation was determined by pre-loading cells with TC, and removing TC from the ^{3}H-lopinavir loading buffer. *Cis*-inhibition was determined for BSP and TC by adding them with ^{3}H-lopinavir to the loading buffer.

Statistical analysis:

Two-way ANOVA was used to compare the uptake of ^{3}H-lopinavir in the presence or absence of inhibitors of ABC transporters at various time intervals. It was also used to compare the same in the efflux experiments. Two-way ANOVA was also used to compare unbound fraction of ^{3}H-lopinavir in the presence or absence of verapamil or ritonavir at 37°C and 4°C (GraphPad Prism version 5).

The uptake or efflux of ^{3}H-lopinavir in the presence or absence of verapamil at 37°C or 4°C was also analyzed using a two-way ANOVA, which was followed by Bonferroni post-tests pairwise comparisons. Efflux of ^{3}H-lopinavir in the presence or absence of GF120918 or ritonavir; and uptake of ^{3}H-lopinavir in the presence or absence of BSP or TC were analyzed by one-way ANOVA with Dunnett's post test (GraphPad Prism version-5). All results are presented as mean ± standard deviation. P-value of 0.05 or less was considered to indicate a statistically significant difference.

CHAPTER 4 RESULTS AND DISCUSSION

PROTEIN BINDING STUDIES:

Protein binding equilibrium was achieved within 16 hours (Figure 2), and non-specific binding was negligible. Fraction unbound for lopinavir in serum obtained from cord blood from term placentae of healthy patients was 0.022 ± 0.011 (mean ± SD, n = 4). The results are similar to unpublished data of Else et al. (0.017 ± 0.010) who reported the protein binding of lopinavir in plasma from cord blood of HIV positive pregnant women at term and on lopinavir/ ritonavir therapy (Else et al., 2007). Fraction unbound for lopinavir in maternal serum samples was 0.0089 ± 0.0012 (mean ± SD, n = 4), which was not significantly different from that observed with cord serum obtained from term placentae ($p= 0.099$). Notably, the variances were significantly different ($p < 0.01$), which may imply more variability in unbound fraction of lopinavir in cord blood. Mean of the fraction unbound determined in triplicate for each of the maternal samples has been tabulated with the patients' clinical data (Table 4). The unbound fraction of ^{3}H-lopinavir did not correlate with the patients' calculated BMIs ($p= 0.087$).

Figure 2: Cord serum binding experiments to determine the time to equilibrium for ^{3}H-lopinavir. Patients 1, 2 and 3 correspond to patients 4212-036, -037 and -038, respectively. Data are presented as mean ± standard deviation.

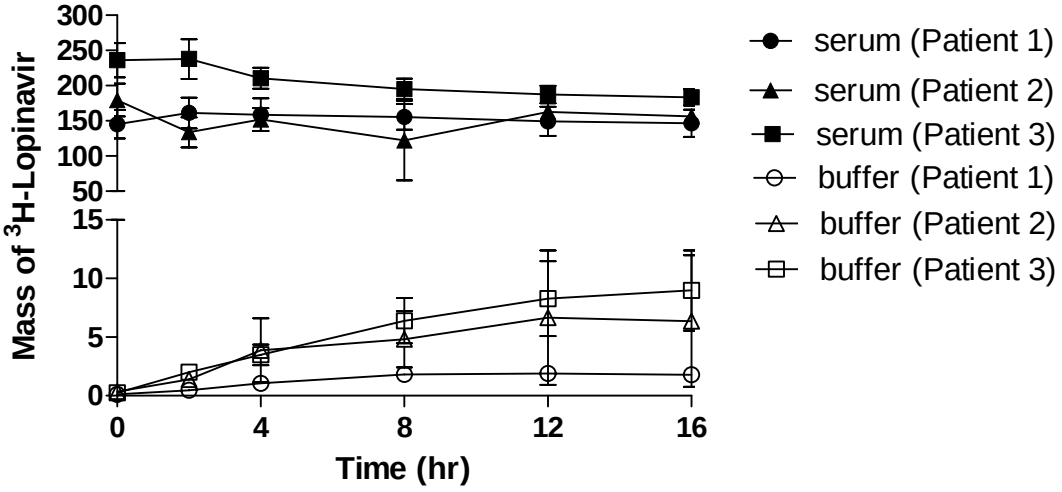


Table 4: Summary of the clinical data of the pregnant patients with their individual mean unbound fraction of ^{3}H-lopinavir. Data are presented as mean ± SD.

Lot number	Age (years)	Gestational Week	Race	Weight (pounds)	Height	BMI	Fraction Unbound for ^{3}H-lopinavir
BC03-122	32	17	Asian	175	5'5"	29.1	0.0077 ± 0.0015
BC03-151	31	27	Caucasian	142	5'3"	25.2	0.0106 ± 0.0011
BC03-182	34	27	African American	266	5'8"	40.4	0.0084 ± 0.0011
BC03-187	19	19	African American	147	5'5"	24.5	0.0090 ± 0.0003

Initial experiments with lopinavir and AAG showed AAG-concentration dependent lopinavir binding, which was consistent with saturability. Experiments with lopinavir and bovine serum albumin (BSA) showed a BSA-concentration dependent lopinavir binding. It was observed that lopinavir was appreciably bound to BSA as well as AAG. However, these initial pilot studies indicated that further studies would have to be carried out with HSA using even wider range of lopinavir and protein concentrations, probably lower HSA concentrations in order to obtain estimates of protein binding kinetic parameters.

Lopinavir binding to 7 μM (0.3 g/L) and 23 μM (1.0 g/L) AAG at varying drug concentrations (0.1-30μM) is shown in Figure 3. The binding of lopinavir to AAG was dependent on protein and drug concentrations. Fraction bound varied from 0.59 ± 0.03 to 0.27 ± 0.01 at 7 μM (0.3 g/L) AAG and from 0.87 ± 0.01 to 0.62 ± 0.03 at 23 μM (1.0 g/L) AAG over a lopinavir concentration range of 0.1-30 μM. It was not possible to determine the binding of lopinavir at 100 μM due to the low solubility of lopinavir. The data obtained for binding of lopinavir at both AAG concentrations were simultaneously fit to Equation (1) with weighting ($1/y^2$). Figure 4 shows the saturation binding curve of lopinavir at 7 μM (0.3 g/L) and 23 μM (1.0 g/L) AAG concentrations in triplicate determinations. K_D was 5.0 ± 1.1 μM and N was 1.2 ± 0.2. Binding of lopinavir to AAG thus appeared saturable.

<u>Figure 3:</u> Lopinavir binding to AAG: Lopinavir binding at varying drug concentrations (0.1-30 μM) to 7 μM AAG (●) and at 0.1-100 μM to 23 μM AAG (□). Data are presented as mean ± standard deviation.

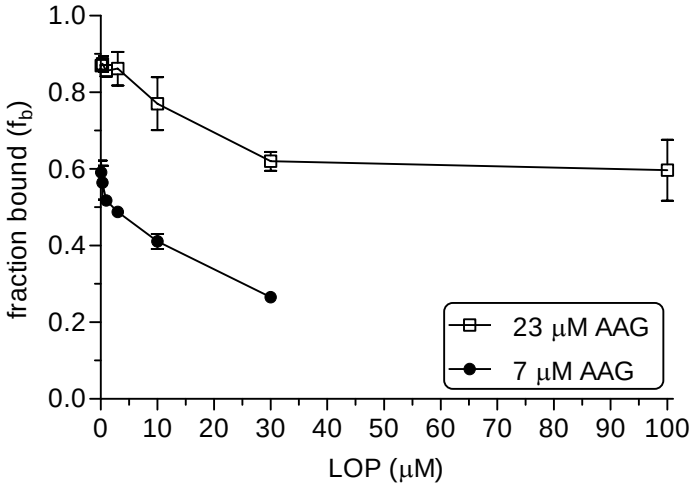
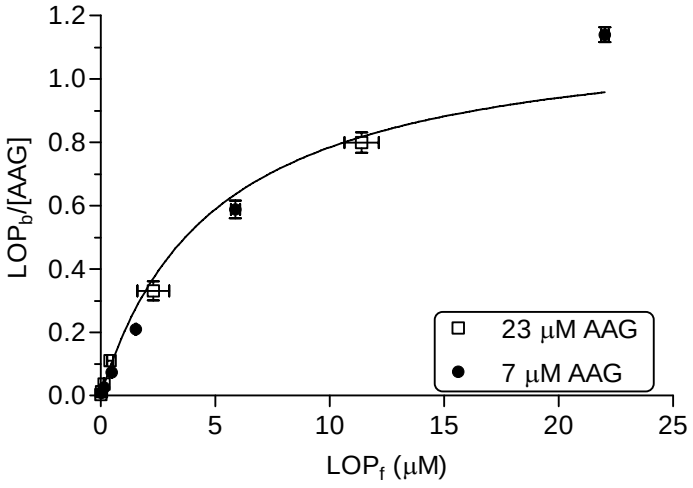
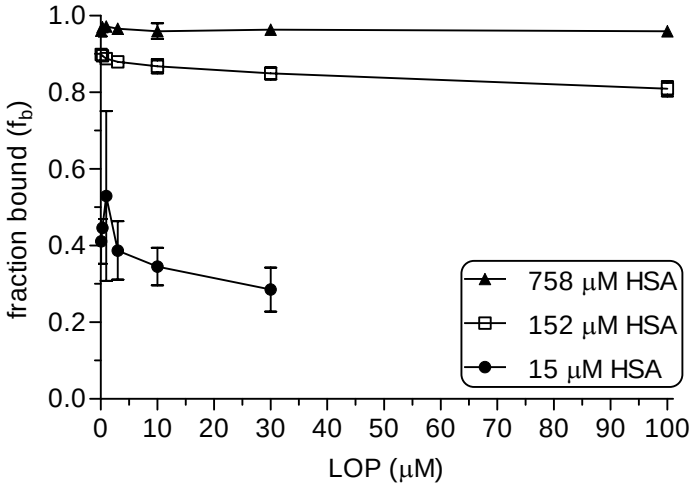


Figure 4: Lopinavir binding to AAG: Bound lopinavir concentrations normalized for AAG concentration as a function of unbound lopinavir concentrations. AAG: 7 μM (●); 23 μM (□). Data are presented as mean ± standard deviation.

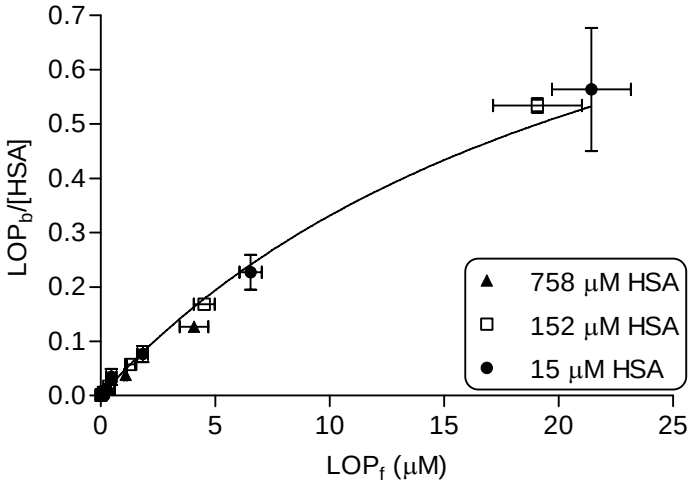


Similar triplicate experiments were carried out at several HSA concentrations (15, 152, or 758 μM; equal to 1.0, 10.0, or 50.0 g/L, respectively). Lopinavir binding to 15, 152 and 758 μM (1.0, 10.0 and 50.0 g/L, respectively) HSA at varying drug concentrations (0.1-100 μM) is shown in Figure 5. In contrast to binding of lopinavir to AAG, binding to HSA was less dependent on lopinavir concentration; however, binding was dependent upon HSA concentration. With HSA, fraction bound ranged from 0.41 ± 0.06 to 0.29 ± 0.06 over a lopinavir concentration range of 0.1-30 μM at 15 μM (1.0 g/L) HSA and from 0.90 ± 0.01 to 0.81 ± 0.02 over a lopinavir concentration range of 0.1-100 μM at 152 μM (10.0 g/L) HSA. It was not possible to determine the binding of lopinavir at 100 μM in the presence of a low HSA concentration (15 μM = 1.0 g/L) due to the low solubility of lopinavir. At 758 μM (50.0 g/L) HSA, fraction bound of lopinavir (0.96 ± 0.02) was independent of lopinavir concentration (0.1-100 μM). The data obtained for binding of lopinavir to varying HSA concentrations were also simultaneously fit to Equation (1) with weighting ($1/y^2$). Figure 6 shows the saturation binding curve of lopinavir at 15, 152, and 758 μM (equal to 1.0, 10.0, and 50.0 g/L) HSA concentrations. K_D was estimated to be 24.3 ± 8.7 μM and N was 1.1 ± 0.4. However, at a physiologic concentration of 758 μM (50.0 g/L) HSA, lopinavir binding was essentially non-saturable.

<u>Figure 5:</u> Lopinavir binding to HSA: Lopinavir binding at varying drug concentrations (0.1-30 μM) to 15 μM HSA (●), and at 0.1-100 μM lopinavir to 152 μM (□) and 758 μM HSA (▲). Data are presented as mean ± standard deviation.



<u>Figure 6:</u> Lopinavir binding to HSA: Bound lopinavir concentrations normalized for HSA concentration as a function of unbound lopinavir concentrations. HSA: 15 μM (●); 152 μM (□); 758 μM (▲). Data are presented as mean ± standard deviation.



The K_D values obtained in the case of experiments with AAG and HSA can be compared to the therapeutic concentrations of lopinavir to determine if the binding is saturable or non-saturable. Lopinavir C_{max} following administration of 400 mg lopinavir with 100 mg ritonavir to healthy male volunteers is 13.5 μM (http://www.fda.gov/cder/foi/nda/2000/21-226_Kaletra_biopharmr_P1.pdf). However, the estimated unbound lopinavir concentration would be approximately 0.2 μM. According to the results obtained above, K_D for lopinavir binding to AAG and HSA of 5.0 ± 1.1 μM and 24.3 ± 8.7 μM, respectively, were much higher than the estimated unbound lopinavir concentration. Thus, lopinavir binding to both AAG and HSA would be non-saturable at therapeutic concentrations.

The data indicate that binding to HSA and AAG can account for the total protein binding of lopinavir by multiplying the expected unbound fractions of lopinavir at those protein concentrations. For the AAG and HSA concentration ranges previously discussed, the predicted lopinavir protein binding would vary between 0.96-0.99 in the maternal serum and 0.86-0.98 in the fetal serum during the course of pregnancy, as protein concentrations in maternal and fetal serum change between 12 and 41 weeks gestation. The range of these predictions includes our observed unbound fractions in both fetal and maternal serum. The trend in the present results toward a higher unbound fraction for lopinavir in fetal serum is consistent with the higher unbound fraction in cord blood reported for indinavir and saquinavir (Sudhakaran et al., 2007).

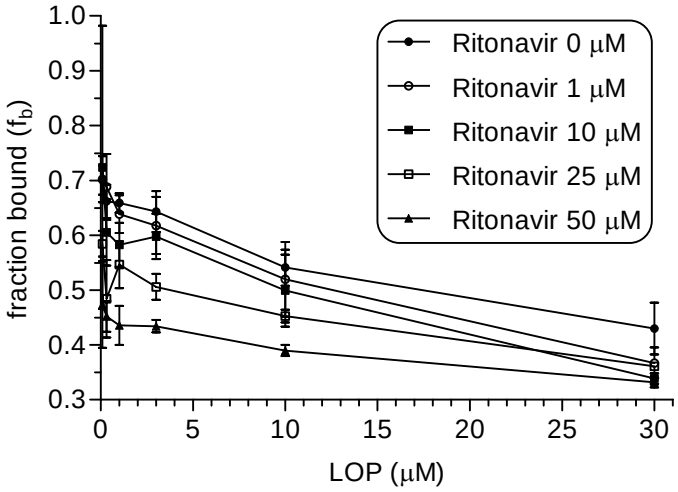
The results show that protein binding of lopinavir is characterized by saturable binding to AAG and non-saturable binding to albumin at physiologic protein

concentrations. Although HSA binds lopinavir with a somewhat lower affinity and both proteins appear to bind lopinavir at a single site, the physiologic concentration of HSA is much greater resulting in a much higher binding capacity. As a result, HSA would contribute more to the high degree of total plasma protein binding of lopinavir.

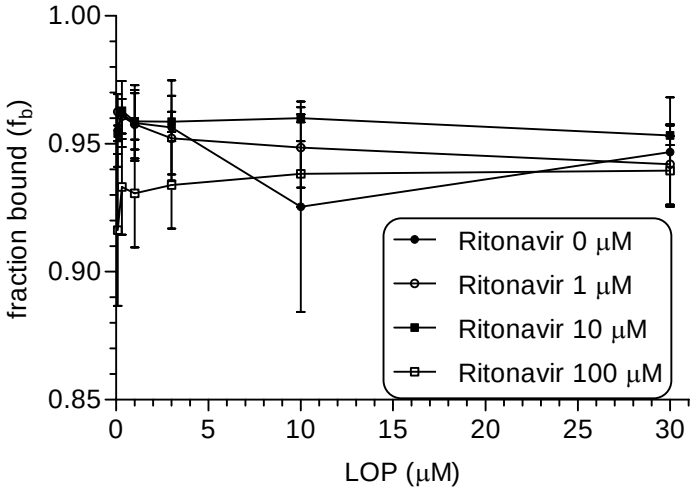
Effect of ritonavir on lopinavir binding to AAG and HSA

Figure 7 shows the lopinavir binding to 9 μM (0.38 g/L) AAG at various lopinavir concentrations (0.1-30 μM) in the presence of ritonavir (0, 1, 10, 25, or 50 μM). Lopinavir binding to AAG was significantly displaced by ritonavir only at 50 μM but not lower concentrations; it was not possible to obtain a reliable estimate of K_i. Figure 8 shows lopinavir binding to 530 μM (35 g/L) HSA at various lopinavir concentrations (0.1-30 μM) in the presence of ritonavir (0, 1, 10, or 100 μM). Ritonavir (1, 10 μM) had no apparent effect on unbound fraction of lopinavir in the presence of 530 μM (35 g/L) HSA. Only in the presence of 100 μM ritonavir (but not lower concentrations), lopinavir binding to HSA was significantly altered.

<u>Figure 7:</u> Effect of ritonavir on lopinavir binding to AAG: Lopinavir binding to 9 μM AAG at various concentrations of lopinavir (0.1-30 μM) in the presence of ritonavir (0, 1, 10, 25, or 50 μM; ●, ○, ■, □, ▲, respectively). Data are presented as mean ± standard deviation and were analyzed by ANOVA with Dunnett's post-test ($p < 0.05$).



<u>Figure 8:</u> Effect of ritonavir on lopinavir binding to HSA: Lopinavir binding to 530 μM HSA at various concentrations of lopinavir (0.1-30 μM) in the presence of ritonavir (0, 1, 10, or 100 μM; ●, ○, ■, □, respectively). Data are presented as mean ± standard deviation and were analyzed by ANOVA with Dunnett's post-test ($p < 0.05$).



Therefore, ritonavir which is co-administered with lopinavir to HIV infected pregnant women may not act to displace lopinavir from binding to AAG or HSA at its therapeutically relevant concentrations (C_{max} = 0.83 μM, C_{trough} = 0.30 μM) (Murphy et al., 2001).

BeWo CELL CULTURE STUDIES:

There were many preliminary time course uptake as well as efflux experiments that were performed with ^{3}H-lopinavir using the BeWo cell line. The results are in Appendix IV. The results consistently suggested that verapamil (100 μM) either inhibited the uptake of ^{3}H-lopinavir (0.32 μM) or stimulated its efflux. However, some technical issues (cells not washed before adding the lysis buffer, stock buffers not analyzed) may hinder the interpretation of these results, and the preliminary results have therefore been included as an appendix.

Because there was significantly higher ^{3}H-lopinavir observed in the stock solution with sodium orthovanadate compared to the stock without sodium orthovanadate (results in Appendix IV), there was a possibility of non-specific binding of ^{3}H-lopinavir to the plastic, which may have been displaced by sodium orthovanadate. To confirm these results, an experiment on 12-well plates without the cells was carried out with ^{3}H-lopinavir (0.32 μM) ± verapamil (100 μM). It was observed that almost 10-times as much ^{3}H-lopinavir stuck to the plate in the absence of verapamil (28 ± 6 pmols) compared to that in the presence of verapamil (3 ± 1 pmols). Therefore, it was concluded that ^{3}H-lopinavir significantly binds to plastic and verapamil displaces it from these non-specific binding sites.

As lopinavir is a highly plasma protein bound drug, binding experiments using RED were carried out to determine lopinavir binding to placental tissue and BeWo cell pellets. Placental tissue from pregnant women (patients 4212-034, -038, and -040) was homogenized in triplicate to determine any lopinavir binding to the placental tissue. At an average protein concentration of 13 g/L, mean fraction bound of ^{3}H-lopinavir (0.32 μM) to the tissue homogenates was 0.86 ± 0.03. Interestingly, these results correlated well with the ^{3}H-lopinavir protein binding observed with HSA, in which fraction bound of ^{3}H-lopinavir in the presence of 10 g/L HSA was 0.90 ± 0.01 to 0.81 ± 0.02 over a lopinavir concentration range of 0.1-100 μM. Similarly, binding experiments with BeWo cell homogenates showed that ^{3}H-lopinavir is considerably bound to the cell homogenate as well. ^{3}H-lopinavir binding with the cell pellet homogenate was 0.77 ± 0.04 with the protein content estimated to be 3.57 g/L. Extrapolating these results to binding at approximately 10 times higher protein content, the unbound fraction of ^{3}H-lopinavir would be around 0.023, which is similar to that observed with cord serum samples (0.022 ± 0.011).

Because lopinavir binds non-specifically to plastic, BeWo cells, and also to the placental tissue, we added a low concentration of BSA (0.05 %) during the experiments with ^{3}H-lopinavir and the inhibitors of ABC transporters to decrease non-specific binding and to get a better estimate of ^{3}H-lopinavir being taken into the cells and effluxed out of the cells. But the question was where to include 0.05% BSA during the experiments. Two separate efflux experiments without the BeWo cells were carried out to answer this question and to determine any effect of including BSA on lopinavir sticking to the plate. ^{3}H-lopinavir (0.32μM) efflux was studied as previously in the presence or absence of

verapamil (100μM), with 0.05% BSA either in the ^{3}H-lopinavir ± verapamil stock solutions or in the pre-incubation buffer. The results of these experiments indicated that almost 16 times as much ^{3}H-lopinavir still stuck to the plate in the absence of verapamil when BSA was included in the stock solutions. In contrast, BSA in the pre-incubation buffer caused the ^{3}H-lopinavir concentration to be almost equal in the two stock solutions. Therefore, it seemed like a pre-incubation step in the presence of 0.05% BSA was necessary to limit non-specific binding in order to better compare ^{3}H-lopinavir transport in the presence or absence of verapamil or any other inhibitor.

Therefore, based on the above experiments and results obtained, following conditions were thought to be critical while carrying out experiments with ^{3}H-lopinavir:

- BSA (0.05%) should be present throughout the experiments, including the pre-incubation buffers, washing buffers and the stock solutions.
- Stock solutions of ^{3}H-lopinavir should be prepared in glass tubes so as to avoid its non-specific binding to plastic.
- No verapamil or any other inhibitor should be present at the uptake step in an efflux experiment to differentiate between uptake and efflux processes.
- BeWo cells should be washed at least twice with ice-cold buffer containing 0.05% BSA before adding the lysis buffer in an uptake experiment. In an efflux experiment, cells should be washed with ice-cold buffer at least twice after the uptake step and again twice after the efflux step before adding the lysis buffer.

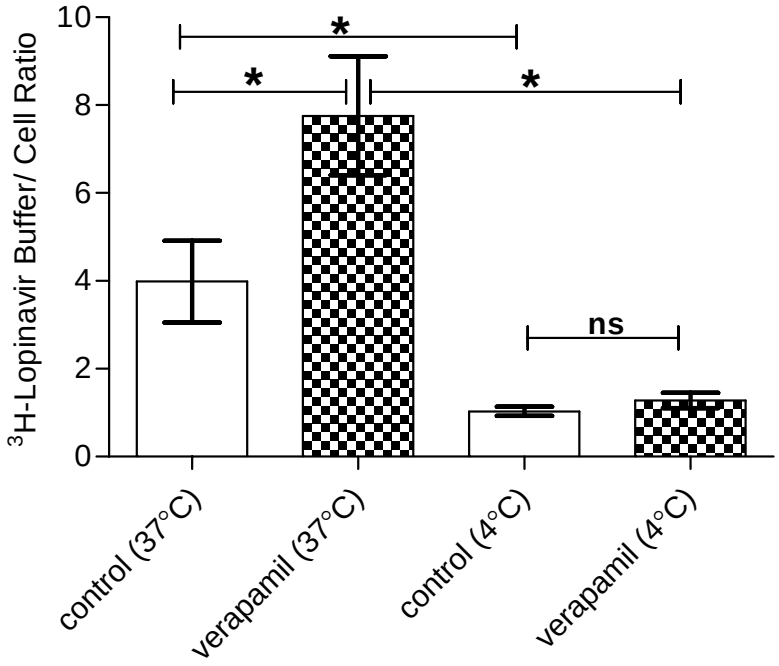
Experiments to differentiate between uptake and binding:

To differentiate between binding and uptake issues, further uptake and efflux experiments were carried out at 37°C and at 4°C, assuming that the transport processes at 4°C would be inhibited and therefore, would help to differentiate between uptake and binding processes. The experiments were carried out as mentioned above, but at two temperatures with control and verapamil treated groups.

Efflux of ^{3}H-lopinavir in BeWo cells:

Next, an efflux experiment with ^{3}H-lopinavir (0.32 μM) and verapamil (100 μM) was carried out and the results obtained are presented in Figure 9. A significantly higher ^{3}H-lopinavir buffer/cell ratio at 37°C in the presence of verapamil shows a stimulation of ^{3}H-lopinavir efflux. This stimulation of ^{3}H-lopinavir efflux observed instead of inhibition by verapamil may be due to ABCC2-mediated transport stimulation in the presence of GSH. This stimulation of ABCC2-mediated efflux by verapamil in the presence of GSH has been reported earlier (Vaidya et al., 2009). However, expression of ABCC2 in BeWo cells is low (Pascolo et al., 2003; Evseenko et al., 2006b), and thus, indicates a possible involvement of a different transporter in lopinavir efflux. The apparent increase in ^{3}H-lopinavir buffer/cell ratio at 37°C in the presence of verapamil would also include any intracellular ^{3}H-lopinavir binding displacement caused by verapamil.

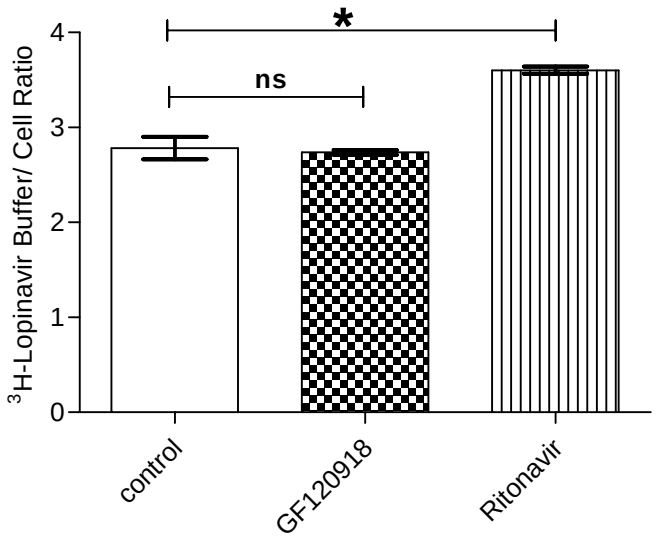
Figure 9: Efflux of ^{3}H-lopinavir (0.32 μM) in the presence or absence of verapamil (100μM) at 37°C or 4°C. Results are presented as mean ± standard deviation and were analyzed using two-way ANOVA followed by Bonferroni post-tests pairwise comparisons. Asterisks indicate significant difference ($p < 0.05$); "ns" indicates that the comparison was not significant.



A significantly lower ^{3}H-lopinavir buffer/cell ratio in the control group at 4°C compared to that observed in the control group at 37°C indicates a temperature-sensitive efflux process. A significantly lower ^{3}H-lopinavir buffer/cell ratio at 4°C compared to that observed at 37°C in the verapamil-treated groups also indicates a temperature-sensitive efflux process. In addition, at 4°C, there was no significant difference between the control and verapamil-treated groups, consistent with minimal intracellular binding displacement by verapamil.

An efflux experiment at 37°C with GF120918 (1 μM), a known ABCB1 inhibitor and with ritonavir (10 μM), known ABCB1, ABCC1, and ABCC2 substrate was carried out and the results are shown in Figure 10. There was a significant increase in ^{3}H-lopinavir buffer/cell ratio in the presence of ritonavir which suggests either inhibition of uptake or stimulation of efflux by ritonavir. Another possibility is an increase in unbound fraction of lopinavir in the cell by intracellular binding displacement which may have caused an increase in the free drug being effluxed out of the cell. There was no significant difference observed between ^{3}H-lopinavir buffer/cell ratio in the presence or absence of GF120918 at an effective concentration for selectively inhibiting ABCB1 (Matsson et al., 2009), suggesting either the lack of ABCB1 expression in BeWo cells or its lack of activity on lopinavir efflux. Other studies in our lab suggest the lack of ABCB1 expression in our BeWo cells (Drew Landsberg, unpublished data).

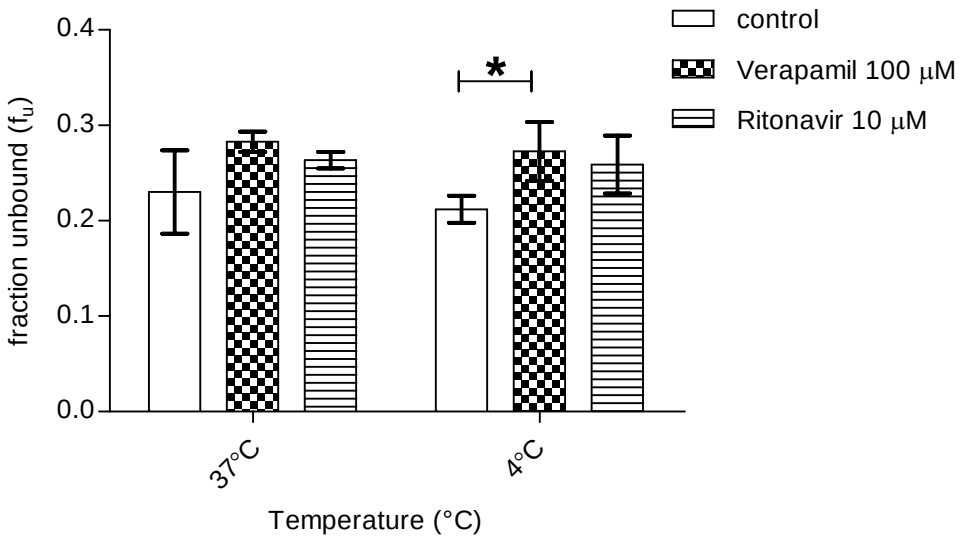
Figure 10: Efflux of ^{3}H-lopinavir (0.32 μM) in the presence or absence of GF120918 (1μM) or ritonavir (10 μM). Results are presented as mean ± standard deviation and were analyzed using one-way ANOVA followed by Dunnett's post-test. Asterisk indicates significant difference ($p < 0.05$); "ns" indicates that the comparison was not significant.



Binding of ^{3}H-lopinavir in BeWo cells:

To determine intracellular binding displacement of ^{3}H-lopinavir by verapamil or ritonavir, and the effects of temperature, a BeWo cell pellet binding experiment was done with ^{3}H-lopinavir in the presence or absence of verapamil (100 μM) or ritonavir (10 μM), as shown in Figure 11. A two-way ANOVA showed that the effect of temperature was not significant ($p > 0.05$). At 37°C, the unbound fraction of ^{3}H-lopinavir alone or in the presence of verapamil or ritonavir did not differ (0.23 ± 0.04, 0.28 ± 0.01, 0.26 ± 0.01, respectively). At 4°C, the unbound fraction of ^{3}H-lopinavir alone (0.21 ± 0.01) was significantly different from that in the presence of verapamil (0.27 ± 0.03) but was not different from that in the presence of ritonavir (0.26 ± 0.03). These results suggest that changes in apparent efflux of lopinavir in the presence of ritonavir or at reduced temperature are not due to displacement of lopinavir from cellular binding. In addition, there was a slight but significant displacement (29%) of lopinavir binding by verapamil at 4°C. In Figure 9 shown earlier, the trend in the ^{3}H-lopinavir buffer-to-cell ratio in the presence of verapamil compared to that in the absence of verapamil at 4°C shows a slight increase in apparent efflux of lopinavir, though this increase was not significant. This, in addition to minimal displacement by verapamil observed above, indicates a major efflux component in lopinavir transport which was inhibited at 4°C (Figure 9).

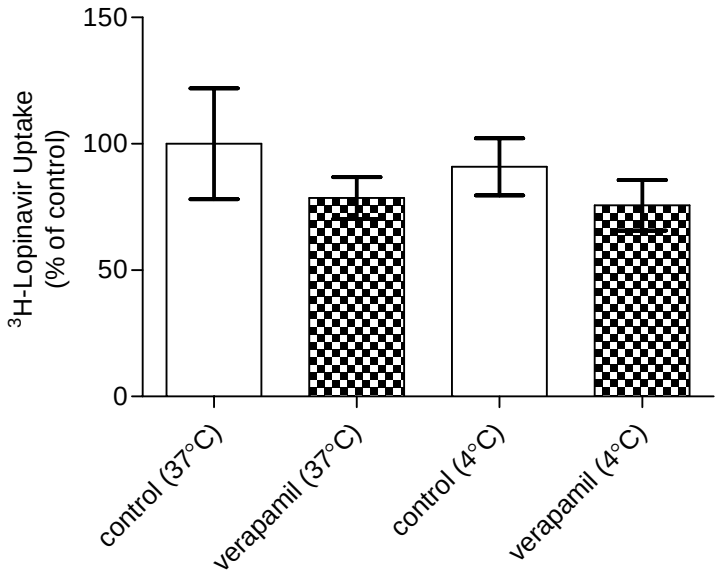
Figure 11: Fraction unbound of ^{3}H-lopinavir (0.32 μM) in the presence or absence of verapamil (100 μM) or ritonavir (10 μM) at 37°C and 4°C. Results are presented as mean ± standard deviation and were analyzed using two-way ANOVA followed by Bonferroni post-tests pairwise comparisons. Asterisk indicates significant difference ($p < 0.05$); other comparisons were not significant.



Uptake of ^{3}H-lopinavir in BeWo cells:

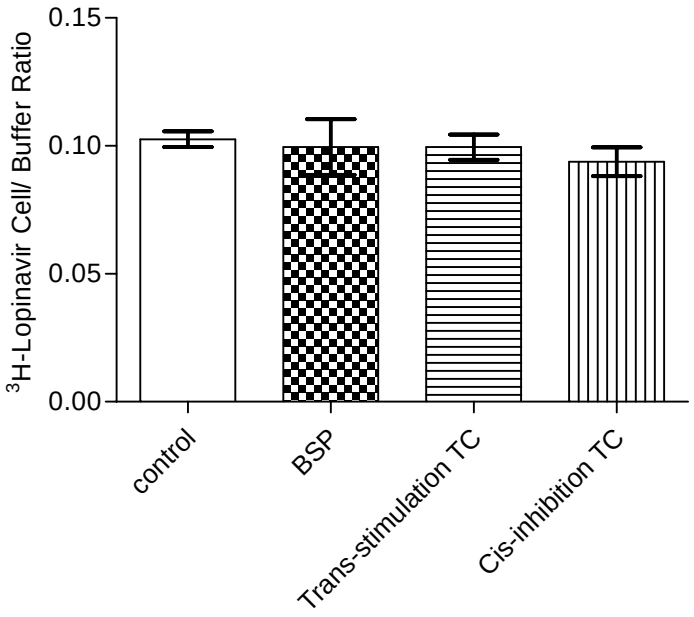
Uptake experiments with ^{3}H-lopinavir (0.32 μM) and verapamil (100 μM) were carried out. Data were pooled from three separate experiments, each with triplicate determinations and the results were expressed as mean ± SEM (% of control). Control ^{3}H-lopinavir cell/ buffer ratio was 0.107 ± 0.024 (mean ± SD). It is reported that inhibitors of ABC transporters like verapamil may also inhibit OATPs (Cvetkovic et al., 1999) and thus, may affect the uptake of drugs into the cells. The authors in this study showed that verapamil at 100 μM caused a 100% inhibition of OATP-mediated ^{14}C-fexofenadine (2 μM) uptake. ^{3}H-lopinavir uptake, however, was not sensitive to verapamil (Figure 12). In addition, the insensitivity of ^{3}H-lopinavir uptake to reduced temperature suggests the involvement of diffusion rather than OATPs.

Figure 12: Uptake of ^{3}H-lopinavir (0.32 μM) in the presence or absence of verapamil (100μM) at 37°C or 4°C. Data were pooled from three separate uptake experiments, each with triplicate determinations and is expressed as Mean ± SEM (% of control). Data were analyzed using two-way ANOVA followed by Bonferroni post-tests pairwise comparisons ($p < 0.05$); none of the comparisons were significant. Control (^{3}H-lopinavir cell/ buffer ratio) = 0.107 ± 0.024 (mean ± SD).



Furthermore, an uptake experiment at 37°C was done with ^{3}H-lopinavir and BSP (50 μM) and TC (100 μM) as OATP inhibitors (Figure 13). BSP (50 μM) and TC (100μM) inhibit OATP1B1, 1B3 and 1A2 (Fischer et al., 2005). BSP also inhibits OATP2B1 (Sai et al., 2006). No significant differences between ^{3}H-lopinavir cell/ buffer ratio between the control, BSP treated and TC treated groups were observed. *Cis*-inhibition with BSP and TC as well as *trans*-stimulation by TC did not change the cell/ buffer ratio compared to control, which suggests that the uptake mechanism(s) for ^{3}H-lopinavir in BeWo cells is not sensitive to BSP or TC. This indicates that the uptake transporter involved in ^{3}H-lopinavir uptake is either an OATP isoform that is not inhibited by BSP or TC or there is an entirely different uptake transporter involved. However, temperature independent uptake observed in Figure 12 suggests another (unknown) mechanism or simple diffusion.

Figure 13: Uptake of ^{3}H-lopinavir (0.32 μM) in the presence or absence of bromosulfophthalein (50 μM) or taurocholate (100 μM) at 37°C. Results are presented as mean ± standard deviation and were analyzed using one-way ANOVA followed by Dunnett's post-test ($p < 0.05$); none of the comparisons were significant.



CHAPTER 5 CONCLUSIONS

Protein binding studies with cord and maternal serum show that fraction unbound for lopinavir in serum obtained from cord blood from term placentae of healthy patients was 0.022 ± 0.011, which was not significantly different from that observed with maternal serum obtained from term placentae (0.0089 ± 0.0012, p= 0.099). Notably, the variances were significantly different ($p < 0.01$), which may imply more variability in unbound fraction of lopinavir in cord blood.

Other protein binding studies show that protein binding of lopinavir is characterized by saturable binding to AAG (K_D = 5.0 ± 1.1 μM, N = 1.2 ± 0.2) and non-saturable binding to albumin at physiologic protein concentrations. Although HSA binds lopinavir with a somewhat lower affinity (K_D = 24.3 ± 8.7 μM, N = 1.1 ± 0.4) and both proteins appear to bind lopinavir at a single site, the physiologic concentration of HSA is much greater resulting in a much higher binding capacity. As a result, HSA would contribute more to the high degree of total plasma protein binding of lopinavir.

The concentration of serum proteins, the number of binding sites, and the apparent K_D determine the extent to which drugs such as lopinavir are bound to proteins. It is anticipated that in the absence of active transport processes, equilibrium would occur for unbound concentrations in fetal and maternal blood for drugs with a long half-life. Thus,

changes in fraction unbound (due to alterations in binding kinetics) in either circulation would change the fetal to maternal serum total drug concentration ratios, while the unbound concentration ratios would presumably remain constant.

Lopinavir binds non-specifically to plastic, BeWo cells, and also to the placental tissue. The BeWo cell line is of human origin and expresses various ABC and OATP transporters similar to the human placental syncytiotrophoblast. The results suggest that verapamil (100μM) stimulated apparent efflux of ^{3}H-lopinavir by two fold, possibly due to ABCC2. In addition, this efflux process was 75% inhibited by reduced temperature (4°C). Ritonavir (10 μM) also stimulated ^{3}H-lopinavir efflux, whereas GF120918 (1 μM) had no effect. Reduced temperature (4°C), verapamil (100 μM) or ritonavir (10 μM) individually did not significantly affect the binding of ^{3}H-lopinavir to cell homogenates. However, slight but significant binding displacement by verapamil at 4°C was observed. ^{3}H-lopinavir uptake was not sensitive to verapamil, BSP, TC or to reduced temperature suggesting uptake involves diffusion rather than OATPs. Based on the results obtained with BeWo cell culture studies, interplay between cellular binding and ABC efflux transporters, in addition to simple diffusion, determines the extent of ^{3}H-lopinavir distribution into BeWo cells.

Further studies considering individual transporters, that is, using a cell line that has been transfected by single transporter genes (like MDCK-ABCB1, MDCK-ABCC1) would complement studies of lopinavir transport in trophoblasts. Finally, mechanistic studies of transplacental transfer of highly protein bound drugs such as lopinavir should consider unbound fetal to maternal concentration ratios as well as cellular binding.

Literature Cited

Literature Cited

Acosta EP, Zorrilla C, Van Dyke R, Bardeguez A, Smith E, Hughes M, Huang S, Pitt J, Watts H and Mofenson L (2001) Pharmacokinetics of saquinavir-SGC in HIV-infected pregnant women. *HIV Clin Trials* **2:**460-465.

Anderson GD (2005) Pregnancy-induced changes in pharmacokinetics: a mechanistic-based approach. *Clin Pharmacokinet* **44:**989-1008.

Atkinson DE, Greenwood SL, Sibley CP, Glazier JD and Fairbairn LJ (2003) Role of MDR1 and MRP1 in trophoblast cells, elucidated using retroviral gene transfer. *Am J Physiol Cell Physiol* **285:**C584-591.

Bode CJ, Jin H, Rytting E, Silverstein PS, Young AM and Audus KL (2006) In Vitro Models for Studying Trophoblast Transcellular Transport, in: *Placenta and Trophoblast: Methods and Protocols*, pp 225-239, Humana Press.

Boffito M, Sciole K, Raiteri R, Bonora S, Hoggard PG, Back DJ and Di Perri G (2002) Alpha 1-acid glycoprotein levels in human immunodeficiency virus-infected subjects on antiretroviral regimens. *Drug Metab Dispos* **30:**859-860.

Brangi M, Litman T, Ciotti M, Nishiyama K, Kohlhagen G, Takimoto C, Robey R, Pommier Y, Fojo T and Bates SE (1999) Camptothecin resistance: role of the ATP-binding cassette (ABC), mitoxantrone-resistance half-transporter (MXR), and potential for glucuronidation in MXR-expressing cells. *Cancer Res* **59:**5938-5946.

Briz O, Serrano MA, MacIas RI, Gonzalez-Gallego J and Marin JJ (2003) Role of organic anion-transporting polypeptides, OATP-A, OATP-C and OATP-8, in the human placenta-maternal liver tandem excretory pathway for foetal bilirubin. *Biochem J* **371:**897-905.

Ceckova-Novotna M, Pavek P and Staud F (2006) P-glycoprotein in the placenta: expression, localization, regulation and function. *Reprod Toxicol* **22:**400-410.

Chappuy H, Treluyer JM, Jullien V, Dimet J, Rey E, Fouche M, Firtion G, Pons G and Mandelbrot L (2004a) Maternal-fetal transfer and amniotic fluid accumulation of nucleoside analogue reverse transcriptase inhibitors in human immunodeficiency virus-infected pregnant women. *Antimicrob Agents Chemother* **48:**4332-4336.

Chappuy H, Treluyer JM, Rey E, Dimet J, Fouche M, Firtion G, Pons G and Mandelbrot L (2004b) Maternal-fetal transfer and amniotic fluid accumulation of protease inhibitors in pregnant women who are infected with human immunodeficiency virus. *Am J Obstet Gynecol* **191:**558-562.

Choo EF, Leake B, Wandel C, Imamura H, Wood AJ, Wilkinson GR and Kim RB (2000) Pharmacological inhibition of P-glycoprotein transport enhances the distribution of HIV-1 protease inhibitors into brain and testes. *Drug Metab Dispos* **28:**655-660.

Chu CY, Singla VP, Wang HP, Sweet B and Lai LT (1981) Plasma alpha 1-acid glycoprotein levels in pregnancy. *Clin Chim Acta* **112:**235-240.

Conway B (2009) HAART in treatment-experienced patients in the 21st century: the audacity of hope. *Future Virology* **4:**39-41.

Cvetkovic M, Leake B, Fromm MF, Wilkinson GR and Kim RB (1999) OATP and P-glycoprotein transporters mediate the cellular uptake and excretion of fexofenadine. *Drug Metab Dispos* **27:**866-871.

Denson D, Coyle D, Thompson G and Myers J (1984) Alpha 1-acid glycoprotein and albumin in human serum bupivacaine binding. *Clin Pharmacol Ther* **35:**409-415.

Drewe J, Gutmann H, Fricker G, Torok M, Beglinger C and Huwyler J (1999) HIV protease inhibitor ritonavir: a more potent inhibitor of P-glycoprotein than the cyclosporine analog SDZ PSC 833. *Biochem Pharmacol* **57:**1147-1152.

Else L, Dickinson L, Bell C, Douglas M, Back D, Khoo S and Taylor G (2007) Total and unbound lopinavir pharmacokinetics during pregnancy and at delivery in HIV-infected women., in: *8th International Workshop on Clinical Pharmacology of HIV Infection*, Budapest.

Evseenko D, Paxton JW and Keelan JA (2006a) Active transport across the human placenta: impact on drug efficacy and toxicity. *Expert Opin Drug Metab Toxicol* **2:**51-69.

Evseenko DA, Paxton JW and Keelan JA (2006b) ABC drug transporter expression and functional activity in trophoblast-like cell lines and differentiating primary trophoblast. *Am J Physiol Regul Integr Comp Physiol* **290:**R1357-1365.

Fischer WJ, Altheimer S, Cattori V, Meier PJ, Dietrich DR and Hagenbuch B (2005) Organic anion transporting polypeptides expressed in liver and brain mediate uptake of microcystin. *Toxicol Appl Pharmacol* **203:**257-263.

Ford J, Khoo SH and Back DJ (2004) The intracellular pharmacology of antiretroviral protease inhibitors. *J Antimicrob Chemother* **54:**982-990.

Ganrot PO (1972) Variation of the concentrations of some plasma proteins in normal adults, in pregnant women and in newborns. *Scand J Clin Lab Invest Suppl* **124:**83-88.

Gupta A, Zhang Y, Unadkat JD and Mao Q (2004) HIV protease inhibitors are inhibitors but not substrates of the human breast cancer resistance protein (BCRP/ABCG2). *J Pharmacol Exp Ther* **310:**334-341.

Gutmann H, Fricker G, Drewe J, Toeroek M and Miller DS (1999) Interactions of HIV protease inhibitors with ATP-dependent drug export proteins. *Mol Pharmacol* **56:**383-389.

Hagenbuch B (2007) Cellular entry of thyroid hormones by organic anion transporting polypeptides. *Best Pract Res Clin Endocrinol Metab* **21:**209-221.

Hoggard PG and Owen A (2003) The mechanisms that control intracellular penetration of the HIV protease inhibitors. *J Antimicrob Chemother* **51:**493-496.

Huisman MT, Smit JW, Crommentuyn KM, Zelcer N, Wiltshire HR, Beijnen JH and Schinkel AH (2002) Multidrug resistance protein 2 (MRP2) transports HIV protease inhibitors, and transport can be enhanced by other drugs. *Aids* **16:**2295-2301.

Huisman MT, Smit JW, Wiltshire HR, Hoetelmans RM, Beijnen JH and Schinkel AH (2001) P-glycoprotein limits oral availability, brain, and fetal penetration of saquinavir even with high doses of ritonavir. *Mol Pharmacol* **59:**806-813.

Janneh O, Hartkoorn RC, Jones E, Owen A, Ward SA, Davey R, Back DJ and Khoo SH (2008) Cultured CD4T cells and primary human lymphocytes express hOATPs: intracellular accumulation of saquinavir and lopinavir. *Br J Pharmacol* **155:**875-883.

Jones K, Hoggard PG, Khoo S, Maher B and Back DJ (2001a) Effect of alpha1-acid glycoprotein on the intracellular accumulation of the HIV protease inhibitors saquinavir, ritonavir and indinavir in vitro. *Br J Clin Pharmacol* **51:**99-102.

Jones K, Hoggard PG, Sales SD, Khoo S, Davey R and Back DJ (2001b) Differences in the intracellular accumulation of HIV protease inhibitors in vitro and the effect of active transport. *Aids* **15:**675-681.

Jonker JW, Smit JW, Brinkhuis RF, Maliepaard M, Beijnen JH, Schellens JH and Schinkel AH (2000) Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. *J Natl Cancer Inst* **92:**1651-1656.

Khoo SH, Hoggard PG, Williams I, Meaden ER, Newton P, Wilkins EG, Smith A, Tjia JF, Lloyd J, Jones K, Beeching N, Carey P, Peters B and Back DJ (2002) Intracellular accumulation of human immunodeficiency virus protease inhibitors. *Antimicrob Agents Chemother* **46:**3228-3235.

Kim RB (2003) Organic anion-transporting polypeptide (OATP) transporter family and drug disposition. *Eur J Clin Invest* **33 Suppl 2:**1-5.

Kis O, Zastre JA, Otting A, Ramaswamy M and Bendayan R (2008) Interactions of HIV-1 protease inhibitors with the OATP2B1 influx transporter in Caco-2 cells, in: *AAPS Annual Meeting*, Atlanta, Georgia.

Krauer B, Dayer P and Anner R (1984) Changes in serum albumin and alpha 1-acid glycoprotein concentrations during pregnancy: an analysis of fetal-maternal pairs. *Br J Obstet Gynaecol* **91:**875-881.

Kutscher H, Su Y and Sinko P (2005) Human Organic Anion-Transporting Polypeptide OATP-C (SLC21A6) is an Uptake Transporter of Saquinavir in Microinjected Xenopus laevis Oocytes, in: *AAPS Annual Meeting*, Nashville, Tennessee.

Langmann T, Mauerer R, Zahn A, Moehle C, Probst M, Stremmel W and Schmitz G (2003) Real-time reverse transcription-PCR expression profiling of the complete human ATP-binding cassette transporter superfamily in various tissues. *Clin Chem* **49:**230-238.

Lankas GR, Wise LD, Cartwright ME, Pippert T and Umbenhauer DR (1998) Placental P-glycoprotein deficiency enhances susceptibility to chemically induced birth defects in mice. *Reprod Toxicol* **12:**457-463.

Laurell CB (1968) Orosomucoid and alpha 1-antitrypsin in maternal and fetal sera at parturition. *Scand J Clin Lab Invest* **21:**136-138.

Libman H and Makadon HJ (2007) Transmission, Pathogenesis, and Natural History, in: *HIV*, pp 1-34, American College of Physicians.

Loebstein R, Lalkin A and Koren G (1997) Pharmacokinetic changes during pregnancy and their clinical relevance. *Clin Pharmacokinet* **33:**328-343.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:**265-275.

Magnarin M, Rosati A, Iudicibus SD, Bartoli F and Decorti G (2008) Role of ABC Transporters in the BeWo Trophoblast Cell Line. *Toxicology Mechanisms and Methods* **18:**763-769.

Mandelbrot L, Peytavin G, Firtion G and Farinotti R (2001) Maternal-fetal transfer and amniotic fluid accumulation of lamivudine in human immunodeficiency virus-infected pregnant women. *Am J Obstet Gynecol* **184:**153-158.

Mao Q and Unadkat JD (2005) Role of the breast cancer resistance protein (ABCG2) in drug transport. *Aaps J* **7:**E118-133.

Marzolini C and Kim RB (2005) Placental transfer of antiretroviral drugs. *Clin Pharmacol Ther* **78:**118-122.

Marzolini C, Rudin C, Decosterd LA, Telenti A, Schreyer A, Biollaz J and Buclin T (2002) Transplacental passage of protease inhibitors at delivery. *Aids* **16:**889-893.

Mathias AA, Maggio-Price L, Lai Y, Gupta A and Unadkat JD (2006) Changes in pharmacokinetics of anti-HIV protease inhibitors during pregnancy: the role of CYP3A and P-glycoprotein. *J Pharmacol Exp Ther* **316:**1202-1209.

Matsson P, Pedersen JM, Norinder U, Bergstrom CA and Artursson P (2009) Identification of Novel Specific and General Inhibitors of the Three Major Human ATP-Binding Cassette Transporters P-gp, BCRP and MRP2 Among Registered Drugs. *Pharm Res*.

Meaden ER, Hoggard PG, Newton P, Tjia JF, Aldam D, Cornforth D, Lloyd J, Williams I, Back DJ and Khoo SH (2002) P-glycoprotein and MRP1 expression and reduced ritonavir and saquinavir accumulation in HIV-infected individuals. *J Antimicrob Chemother* **50:**583-588.

Meyer Zu Schwabedissen HE, Grube M, Heydrich B, Linnemann K, Fusch C, Kroemer HK and Jedlitschky G (2005) Expression, localization, and function of MRP5 (ABCC5), a transporter for cyclic nucleotides, in human placenta and cultured human trophoblasts: effects of gestational age and cellular differentiation. *Am J Pathol* **166:**39-48.

Mikkaichi T, Suzuki T, Tanemoto M, Ito S and Abe T (2004) The organic anion transporter (OATP) family. *Drug Metab Pharmacokinet* **19:**171-179.

Miller DS, Nobmann SN, Gutmann H, Toeroek M, Drewe J and Fricker G (2000) Xenobiotic transport across isolated brain microvessels studied by confocal microscopy. *Mol Pharmacol* **58:**1357-1367.

Mirochnick M, Dorenbaum A, Holland D, Cunningham-Schrader B, Cunningham C, Gelber R, Mofenson L, Culnane M, Connor J and Sullivan JL (2002) Concentrations of protease inhibitors in cord blood after in utero exposure. *Pediatr Infect Dis J* **21:**835-838.

Mitra P and Audus KL (2008) In Vitro Models and Multidrug Resistance Mechanisms of the Placental Barrier, in: *Drug Absorption Studies*, pp 368-396, Springer US.

Molsa M, Heikkinen T, Hakkola J, Hakala K, Wallerman O, Wadelius M, Wadelius C and Laine K (2005) Functional role of P-glycoprotein in the human blood-placental barrier. *Clin Pharmacol Ther* **78:**123-131.

Moodley J, Moodley D, Pillay K, Coovadia H, Saba J, van Leeuwen R, Goodwin C, Harrigan PR, Moore KH, Stone C, Plumb R and Johnson MA (1998) Pharmacokinetics and antiretroviral activity of lamivudine alone or when coadministered with zidovudine in human immunodeficiency virus type 1-infected pregnant women and their offspring. *J Infect Dis* **178:**1327-1333.

Murphy RL, Brun S, Hicks C, Eron JJ, Gulick R, King M, White AC, Jr., Benson C, Thompson M, Kessler HA, Hammer S, Bertz R, Hsu A, Japour A and Sun E (2001) ABT-378/ritonavir plus stavudine and lamivudine for the treatment of antiretroviral-naive adults with HIV-1 infection: 48-week results. *Aids* **15:**F1-9.

Nagashige M, Ushigome F, Koyabu N, Hirata K, Kawabuchi M, Hirakawa T, Satoh S, Tsukimori K, Nakano H, Uchiumi T, Kuwano M, Ohtani H and Sawada Y (2003) Basal membrane localization of MRP1 in human placental trophoblast. *Placenta* **24:**951-958.

Nation RL (1981) Meperidine binding in maternal and fetal plasma. *Clin Pharmacol Ther* **29:**472-479.

Niemi M (2007) Role of OATP transporters in the disposition of drugs. *Pharmacogenomics* **8:**787-802.

Olson DP, Scadden DT, D'Aquila RT and De Pasquale MP (2002) The protease inhibitor ritonavir inhibits the functional activity of the multidrug resistance related-protein 1 (MRP-1). *Aids* **16:**1743-1747.

Pascolo L, Fernetti C, Pirulli D, Crovella S, Amoroso A and Tiribelli C (2003) Effects of maturation on RNA transcription and protein expression of four MRP genes in human placenta and in BeWo cells. *Biochem Biophys Res Commun* **303:**259-265.

Patel P, Weerasekera N, Hitchins M, Boyd CA, Johnston DG and Williamson C (2003) Semi quantitative expression analysis of MDR3, FIC1, BSEP, OATP-A, OATP-C,OATP-D, OATP-E and NTCP gene transcripts in 1st and 3rd trimester human placenta. *Placenta* **24:**39-44.

Pavek P, Staud F, Fendrich Z, Sklenarova H, Libra A, Novotna M, Kopecky M, Nobilis M and Semecky V (2003) Examination of the functional activity of P-glycoprotein in the rat placental barrier using rhodamine 123. *J Pharmacol Exp Ther* **305:**1239-1250.

Profit L, Eagling VA and Back DJ (1999) Modulation of P-glycoprotein function in human lymphocytes and Caco-2 cell monolayers by HIV-1 protease inhibitors. *Aids* **13:**1623-1627.

Raynes J (1982) Variations in the relative proportions of microheterogeneous forms of plasma glycoproteins in pregnancy and disease. *Biomed Pharmacother* **36:**77-86.

Roberts SS, Martinez M, Covington DLD, Rode RA, Pasley MV and Woodward WC (2009) Lopinavir/Ritonavir in Pregnancy. *J Acquir Immune Defic Syndr*.

Sai Y, Kaneko Y, Ito S, Mitsuoka K, Kato Y, Tamai I, Artursson P and Tsuji A (2006) Predominant contribution of organic anion transporting polypeptide OATP-B (OATP2B1) to apical uptake of estrone-3-sulfate by human intestinal Caco-2 cells. *Drug Metab Dispos* **34:**1423-1431.

Sato K, Sugawara J, Sato T, Mizutamari H, Suzuki T, Ito A, Mikkaichi T, Onogawa T, Tanemoto M, Unno M, Abe T and Okamura K (2003) Expression of organic anion transporting polypeptide E (OATP-E) in human placenta. *Placenta* **24:**144-148.

Schuetz JD, Connelly MC, Sun D, Paibir SG, Flynn PM, Srinivas RV, Kumar A and Fridland A (1999) MRP4: A previously unidentified factor in resistance to nucleoside-based antiviral drugs. *Nat Med* **5:**1048-1051.

Serrano MA, Macias RI, Briz O, Monte MJ, Blazquez AG, Williamson C, Kubitz R and Marin JJ (2007) Expression in human trophoblast and choriocarcinoma cell lines, BeWo, Jeg-3 and JAr of genes involved in the hepatobiliary-like excretory function of the placenta. *Placenta* **28:**107-117.

Shallcross V, Hartkoorn R, Egan D, Kwan WS, Khoo S, Back D and Owen A (2008a) Influence of SLCO1B1 521T>C polymorphism on Lopinavir plasma concentrations from the Liverpool TDM Registry, in: *9th International Workshop on Clinical Pharmacology of HIV Therapy*, New Orleans.

Shallcross VL, Kwan WS, Hartkoorn R, Mahunga T, Khoo S, Back D and Owen A (2008b) Lopinavir is a substrate of SLCO1A2. The 516A>C and 38T>C polymorphisms do not influence lopinavir plasma concentrations., in: *9th International Congress on Drug Therapy in HIV Infection*, Glasgow.

Smit JW, Huisman MT, van Tellingen O, Wiltshire HR and Schinkel AH (1999) Absence or pharmacological blocking of placental P-glycoprotein profoundly increases fetal drug exposure. *J Clin Invest* **104:**1441-1447.

Srinivas RV, Middlemas D, Flynn P and Fridland A (1998) Human immunodeficiency virus protease inhibitors serve as substrates for multidrug transporter proteins MDR1 and MRP1 but retain antiviral efficacy in cell lines expressing these transporters. *Antimicrob Agents Chemother* **42:**3157-3162.

St-Pierre MV, Hagenbuch B, Ugele B, Meier PJ and Stallmach T (2002) Characterization of an organic anion-transporting polypeptide (OATP-B) in human placenta. *J Clin Endocrinol Metab* **87:**1856-1863.

St-Pierre MV, Serrano MA, Macias RI, Dubs U, Hoechli M, Lauper U, Meier PJ and Marin JJ (2000) Expression of members of the multidrug resistance protein family in human term placenta. *Am J Physiol Regul Integr Comp Physiol* **279:**R1495-1503.

Sudhakaran S, Rayner CR, Li J, Kong DC, Gude NM and Nation RL (2006) Differential protein binding of indinavir and saquinavir in matched maternal and umbilical cord plasma. *Br J Clin Pharmacol* **63:**315-321.

Sudhakaran S, Rayner CR, Li J, Kong DC, Gude NM and Nation RL (2007) Differential protein binding of indinavir and saquinavir in matched maternal and umbilical cord plasma. *Br J Clin Pharmacol* **63:**315-321.

Sudhakaran S, Rayner CR, Li J, Kong DC, Gude NM and Nation RL (2008) Inhibition of placental P-glycoprotein: impact on indinavir transfer to the foetus. *Br J Clin Pharmacol* **65:**667-673.

Sun M, Kingdom J, Baczyk D, Lye SJ, Matthews SG and Gibb W (2006) Expression of the multidrug resistance P-glycoprotein, (ABCB1 glycoprotein) in the human placenta decreases with advancing gestation. *Placenta* **27:**602-609.

Syme MR, Paxton JW and Keelan JA (2004) Drug transfer and metabolism by the human placenta. *Clin Pharmacokinet* **43:**487-514.

Tammur J, Prades C, Arnould I, Rzhetsky A, Hutchinson A, Adachi M, Schuetz JD, Swoboda KJ, Ptacek LJ, Rosier M, Dean M and Allikmets R (2001) Two new genes from the human ATP-binding cassette transporter superfamily, ABCC11 and ABCC12, tandemly duplicated on chromosome 16q12. *Gene* **273:**89-96.

Ugele B, St-Pierre MV, Pihusch M, Bahn A and Hantschmann P (2003) Characterization and identification of steroid sulfate transporters of human placenta. *Am J Physiol Endocrinol Metab* **284:**E390-398.

Unadkat JD, Wara DW, Hughes MD, Mathias AA, Holland DT, Paul ME, Connor J, Huang S, Nguyen BY, Watts DH, Mofenson LM, Smith E, Deutsch P, Kaiser KA and Tuomala RE (2007) Pharmacokinetics and safety of indinavir in human immunodeficiency virus-infected pregnant women. *Antimicrob Agents Chemother* **51:**783-786.

Ushigome F, Takanaga H, Matsuo H, Yanai S, Tsukimori K, Nakano H, Uchiumi T, Nakamura T, Kuwano M, Ohtani H and Sawada Y (2000) Human placental transport of vinblastine, vincristine, digoxin and progesterone: contribution of P-glycoprotein. *Eur J Pharmacol* **408:**1-10.

Utoguchi N, Chandorkar GA, Avery M and Audus KL (2000) Functional expression of P-glycoprotein in primary cultures of human cytotrophoblasts and BeWo cells. *Reprod Toxicol* **14:**217-224.

Vaidya SS, Walsh SW and Gerk PM (2009) Formation and Efflux of ATP-Binding Cassette Transporter Substrate 2,4-Dinitrophenyl-S-Glutathione from Cultured Human Term Placental Villous Tissue Fragments. *Mol Pharm* (http://www.ncbi.nlm.nih.gov/pubmed/19397308?).

van Heeswijk RP, Khaliq Y, Gallicano KD, Bourbeau M, Seguin I, Phillips EJ and Cameron DW (2004) The pharmacokinetics of nelfinavir and M8 during pregnancy and post partum. *Clin Pharmacol Ther* **76:**588-597.

Vishnuvardhan D, Moltke LL, Richert C and Greenblatt DJ (2003) Lopinavir: acute exposure inhibits P-glycoprotein; extended exposure induces P-glycoprotein. *Aids* **17:**1092-1094.

Washington CB, Wiltshire HR, Man M, Moy T, Harris SR, Worth E, Weigl P, Liang Z, Hall D, Marriott L and Blaschke TF (2000) The disposition of saquinavir in normal and P-glycoprotein deficient mice, rats, and in cultured cells. *Drug Metab Dispos* **28:**1058-1062.

Watts DH (2006) Treating HIV during pregnancy: an update on safety issues. *Drug Saf* **29:**467-490.

Watts DH, Brown ZA, Tartaglione T, Burchett SK, Opheim K, Coombs R and Corey L (1991) Pharmacokinetic disposition of zidovudine during pregnancy. *J Infect Dis* **163:**226-232.

Weiss J, Rose J, Storch CH, Ketabi-Kiyanvash N, Sauer A, Haefeli WE and Efferth T (2007) Modulation of human BCRP (ABCG2) activity by anti-HIV drugs. *J Antimicrob Chemother* **59:**238-245.

Wood M and Wood AJ (1981) Changes in plasma drug binding and alpha 1-acid glycoprotein in mother and newborn infant. *Clin Pharmacol Ther* **29:**522-526.

Yabuuchi H, Shimizu H, Takayanagi S and Ishikawa T (2001) Multiple splicing variants of two new human ATP-binding cassette transporters, ABCC11 and ABCC12. *Biochem Biophys Res Commun* **288:**933-939.

APPENDIX I

IRB APPROVAL AND INFORMED CONSENT FORM

VCU Memo

Virginia Commonwealth University

Office of Research
Office of Research Subjects Protection

BioTechnology Research Park
800 East Leigh Street, Suite 114
P.O. Box 980568
Richmond, Virginia 23298-0568

(804) 827-0868
Fax: (804) 827-1448

DATE: September 25, 2008

TO: Phillip Gerk, Pharm D, PhD
School of Pharmacy
Box 980533

FROM: Lea Ann Hansen, Pharm D LeaAnn Hansen, Pharm D IAB
Chairperson, VCU IRB Panel D
Box 980568

RE: **VCU IRB #: 04212**
Title: Placental Drug Transporters in Pregnancy

On September 24, 2008, this research study was ***approved for continuation*** by expedited review according to 45 CFR 46.108(b) and 45 CFR 46.109(e) and 45 CFR 46.110 Category 3.

PROTOCOL: Placental Drug Transporters in Pregnancy (Version 6: May 7, 2008; received in ORSP 8/28/08)

VCU IRB APPROVED CONSENT/ASSENT FORM (attached):

- Research Subject Information and Consent Form (3 pages; received in ORSP 8/28/08)
- Informacion y Formulario de Consentimiento (3 pages; received in ORSP 8/28/08)

This approval expires on August 31, 2009. Federal Regulations/VCU Policy and Procedures require continuing review prior to continuation of approval past that date. Continuing Review report forms will be mailed to you prior to the scheduled review.

If you have any questions, please contact Dr. Lea Ann Hansen, Chairperson, VCU IRB Panel D, at leaann.hansen@gmail.com or 804-994-2444; or you may contact Aleksandra Baldwin, IRB Coordinator, VCU Office of Research Subjects Protection, at akbaldwin@vcu.edu or 827-1445.

Attachment – Conditions of Approval

Conditions of Approval:

In order to comply with federal regulations, industry standards, and the terms of this approval, the investigator must *(as applicable):*

1. Conduct the research as described in and required by the Protocol.

2. Obtain informed consent from all subjects without coercion or undue influence, and provide the potential subject sufficient opportunity to consider whether or not to participate (unless Waiver of Consent is specifically approved or research is exempt).

3. Document informed consent using only the most recently dated consent form bearing the VCU IRB "APPROVED" stamp (unless Waiver of Consent is specifically approved).

4. Provide non-English speaking patients with a translation of the approved Consent Form in the research participant's first language. The Panel must approve the translated version.

5. Obtain prior approval from VCU IRB before implementing any changes whatsoever in the approved protocol or consent form, unless such changes are necessary to protect the safety of human research participants (e.g., permanent/temporary change of PI, addition of performance/collaborative sites, request to include newly incarcerated participants or participants that are wards of the state, addition/deletion of participant groups, etc.). Any departure from these approved documents must be reported to the VCU IRB immediately as an Unanticipated Problem (see #7).

6. Monitor all problems (anticipated and unanticipated) associated with risk to research participants or others.

7. Report Unanticipated Problems (UPs), including protocol deviations, following the VCU IRB requirements and timelines detailed in VCU IRB WPP VIII-7):

8. Obtain prior approval from the VCU IRB before use of any advertisement or other material for recruitment of research participants.

9. Promptly report and/or respond to all inquiries by the VCU IRB concerning the conduct of the approved research when so requested.

10. All protocols that administer acute medical treatment to human research participants must have an emergency preparedness plan. Please refer to VCU guidance on http:/www.research.vcu.edu/irb/guidance.htm.

11. The VCU IRBs operate under the regulatory authorities as described within:
    a) U.S. Department of Health and Human Services Title 45 CFR 46, Subparts A, B, C, and D (for all research, regardless of source of funding) and related guidance documents.
    b) U.S. Food and Drug Administration Chapter I of Title 21 CFR 50 and 56 (for FDA regulated research only) and related guidance documents.
    c) Commonwealth of Virginia Code of Virginia 32.1 Chapter 5.1 Human Research (for all research).

RESEARCH SUBJECT INFORMATION AND CONSENT FORM

TITLE: Placental Drug Transporters in Pregnancy

VCU IRB PROTOCOL NUMBER: 4212

INVESTIGATORS: Phillip M. Gerk, Pharm.D., Ph.D.
Scott W. Walsh, Ph.D.
Susan M. Lanni, M.D.

SPONSORS: **Jeffress Trust**
National Institutes of Health (NIH)

This consent form may contain words that you do not understand. Please ask the study doctor or the study staff to explain any words or information that you do not clearly understand.

PURPOSE OF THE STUDY:
The purpose of this research study is to study the way that drugs get through the placenta, and how the placenta (also known as the afterbirth) may be different in some pregnancies. You are being asked to participate in this study because you are pregnant, and may meet the study entry requirements.

DESCRIPTION OF THE STUDY and PROCEDURE:
In this study, you are being asked to allow your placental tissues (sometimes called "afterbirth") to be studied in a research lab. Normally these tissues are discarded by the hospital. At the time your baby is delivered, you and your baby will be under your doctor's care as usual. After your placenta comes out, it will be placed on ice and taken to our research lab. The lab will study how certain proteins in your placenta influence the way certain drugs cross the placenta. Your medical chart will be reviewed for information regarding your pregnancy and medication use. No additional samples or involvement is required after your baby is born.

If you decide to be in this research study, you will be asked to sign this consent form after you have had all your questions answered.

RISKS AND DISCOMFORTS
There is no additional risk in participating in this study.

BENEFITS
You will receive no medical benefits from being in this study. However, the information from this research study may lead to a better treatment in the future for people with problems in pregnancy, such as preeclampsia and gestational diabetes, or other conditions that require a pregnant woman to take medications.

COSTS
There are no research related costs involved in your participation in this study.

PAYMENT FOR PARTICIPATION
There will not be any payment for participation.

ALTERNATIVE
This is not a treatment study. Your alternative is not to participate in this study.

CONFIDENTIALITY
Confidentiality of personal information about you – including your medical records and personal research data gathered in connection with this study – will be maintained in a manner consistent with federal and state laws and regulations. Information identifying you will only be on paper, not on any computers. It will be kept secure in a locked filing cabinet to which only the Principal Investigator has a key.

You should know that research data or (medical information if applicable) about you may be reviewed or copied by the sponsor of the research or by Virginia Commonwealth University. Personal information about you might be shared with or copied by authorized officials of the Federal Food and Drug Administration, or the Department of Health and Human Services (if applicable). Although results of this research may be presented at meetings or in publications, identifiable personal information pertaining to participants will not be disclosed.

VOLUNTARY PARTICIPATION AND WITHDRAWAL
Your participation in this study is voluntary. You may decide to not participate in this study. Your decision will not change your future medical care at this site or institution.

QUESTIONS
In the future, you may have questions about your study participation. If you have any questions, contact:

Dr. Phillip M. Gerk
Department of Pharmaceutics
410 N. 12th Street, Room 356C
(804) 828-6321

If you have questions about your rights as a research subject, you may contact:

Office of Research Subjects Protection
Virginia Commonwealth University
800 East Leigh Street, Suite 111
PO Box 980568
Richmond, VA 23298
(804) 828-0868

Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions.

APPROVED

9/24/08 RG/AB

CONSENT

I have been provided with an opportunity to read this consent form carefully. All of the questions that I wish to raise concerning this study have been answered.

By signing this consent form, I have not waived any of the legal rights or benefits, to which I otherwise would be entitled. My signature indicates that I freely consent to participate in this research study.

_____

Subject Name, printed

_____ _____

Subject Signature Date

_____ _____

Legally Authorized Representative Signature Date
(if appropriate)

_____ _____

Witness Signature Date
(Required)

_____ _____

Signature of Person Conducting Informed Consent Date
Discussion

_____ _____

Investigator Signature (if different from above) Date

APPROVED

9/24/08 RG IAB

APPENDIX II

BeWo CELL CULTURE PROTOCOL

Cell culture medium:

The medium was prepared by dissolving the contents of one bottle (13.4 g) DMEM powdered media in 870 mL of distilled water. It was then supplemented with 3.5g sodium bicarbonate, stirred for 20 minutes and pH was adjusted to 7.4 using 1N HCl. 10 mL each of 200 mM L-glutamine, 10.000 U/mL penicillin with 10 mg/mL streptomycin and 10 M nonessential amino acids were added to the media. This was followed by addition of 10% (v/v) FBS and filtering through a 0.22-µm disposable filter in a vertical flow biological safety cabinet. The medium was stored at 4°C and was used within 2 weeks.

BeWo cell revival from liquid nitrogen:

The BeWo cells were grown and cultured according to the protocol previously described by Bode et al. (Bode et al., 2006), except for a few minor modifications, which are described below in brief:

1. BeWo cell culture medium (DMEM with 10% FBS) and PBS (1X) were warmed to 37°C. 10X PBS, pH 7.4 was obtained from Quality Biological Inc., catalog number QBI119069101.

2. A vial of frozen BeWo cells was removed from a liquid nitrogen storage tank and the cells were thawed in a 37°C water bath.
3. After the cells were thawed, the cell suspension (1.5-2 mL) was immediately transferred to a 15 mL falcon tube and 5 mL of cold media (4°C) was added dropwise to it.
4. The cells were centrifuged to pellet at 300 G for 8 minutes at room temperature (25°C) using Avanti J-E Centrifuge (Beckman Coulter).
5. The supernatant was aspirated and the cell pellet was resuspended in 2 mL of media.
6. The entire cell suspension was placed into a 25 cm^2 tissue culture flask and another 6 mL of warm media was added to it. The flask was labeled with the cell line, passage number, and date.
7. The cells were grown in a 37°C incubator supplied with 5% CO_2 and 95% relative humidity. All procedures were performed with aseptic techniques.
8. When the cells reached 90-95% confluence, the monolayer was treated with trypsin to detach and disperse the cells, as follows.
9. The media from the 25 cm^2 tissue culture flask was aspirated using a sterile Pasteur pipet.
10. The flask was washed with 5 mL of PBS twice in order to remove serum.
11. The cells were detached from the 25 cm^2 flask by incubating them with 5 mL of 1X trypsin-EDTA solution (10X trypsin from Invitrogen, catalog number 15400054) in PBS for 30 seconds.

12. All but few drops (approximately 1 ml) of the liquid were then aspirated off and the flask was placed in the 37°C incubator for approximately 3 minutes.
13. The 25 cm^2 flask was taken out of the incubator and the cell layer examined under the microscope to ensure that the cells were detached from the substratum and "balled up."
14. The cells were then suspended uniformly in 12 mL of media and the entire cell suspension was transferred to seed cells in a 75 cm^2 tissue culture flask. The flask was then labeled with the cell line, passage number, and date.
15. When the cells became 90-95% confluent, the cells were passaged to a 150 cm^2 tissue culture flask.
16. The cells were detached and dispersed as described earlier for the cells passaged to a 75 cm^2 flask, except about 10 mL of the trypsin-EDTA solution was used.
17. The cells were suspended uniformly in 10 mL of media and 5 mL of such cell suspension was seeded into one 150 cm^2 tissue culture flask, which already had 20 mL of media in it. The flask was labeled with the cell line, passage number, and date.
18. Subsequent passages were performed as follows.

<u>BeWo cell passage for cells growing on a 150 cm^2 flask:</u>

1. Media from the flask of cells to be passaged was aspirated and the flask was washed with 10 mL of PBS twice.

2. The cells were detached and dispersed by incubating them with trypsin-EDTA solution (14 mL of 1X trypsin-EDTA in PBS) for 30 seconds. All but few drops of the liquid were aspirated off and the flask was placed in the 37°C incubator for approximately 3 minutes. The cell detachment was visually confirmed.
3. 10 mL of the medium was added in the flask to stop trypsinization. The cells were uniformly suspended and the entire cell suspension was transferred into a 50 mL falcon tube already containing 15 mL of warm media.
4. The cells were centrifuged to pellet at 300 G for 8 minutes at room temperature using Avanti J-E Centrifuge (Beckman Coulter) in JS 5.3 rotor.
5. The supernatant media was aspirated and the cell pellet was resuspended in 10mL of the medium.
6. 2.5 mL of this cell suspension was seeded to each 150 cm^2 tissue culture flask, which already had 23 mL of media in it.
7. One was added to the passage number shown on the initial frozen vial from liquid nitrogen. The flask was labeled with the cell line, passage number, and date.
8. The cells were fed every other day subsequently.

<u>BeWo cell storage:</u>

1. The cell pellet was obtained from trypsinization of a 150 cm^2 flask.
2. The cells were suspended in 10 mL of DMEM media, the cell density determined by using the trypan blue (Sigma Aldrich, catalog number T8154-100mL) exclusion

method, and a cell suspension (11 mL) with 1 X 10^6 cells/mL in DMEM containing 10% DMSO was created.

3. 1 mL of cell suspension was transferred to each pre-labeled tissue culture vials appropriate for deep freezing.
4. The cells were frozen by placing in a freezing canister and placing in a -80°C freezer for 24 hours. Cell vials were then transferred to a liquid nitrogen storage container for long term storage.

APPENDIX III

LOWRY PROTEIN ASSAY

This assay was based on the method by Lowry et al. (Lowry et al., 1951).

1. Solutions A (2% Na_2CO_3 in 0.1 N NaOH), B_1 (1% $CuSO_4.5H_2O$ in H_2O), and B_2 (2% KNa tartarate in H_2O) were mixed in the proportion A = 98 mL, B_1 = 1 mL, and B_2 = 1mL. This was called Reagent 1.
2. Bovine serum albumin, fraction V (BSA) was added as the standard at volumes 0, 5, 10, 20, 40, 60, 80 µL. 20 µL PBS was added to each and the total volume was brought to 500µL.
3. Samples: To 2.5, 5, 10 µL of the samples (cell or tissue homogenates), 20 µL of PBS was added, followed by 480 µL of water.
4. 2.5 mL of reagent 1 was added to each tube and was vortexed each time.
5. This was let stand at room temperature for 10 minutes.
6. 250 µL of 1N Folin Ciocolteu's reagent (2N solution was diluted 1:1 with water) was added to each tube and vortexed each time.
7. The samples were let stand at room temperature for 30 minutes before reading at 660 nm in a spectrophotometer.

APPENDIX IV

RESULTS OF THE PRELIMINARY EXPERIMENTS PERFORMED WITH ^{3}H-LOPINAVIR USING BeWo CELL LINE

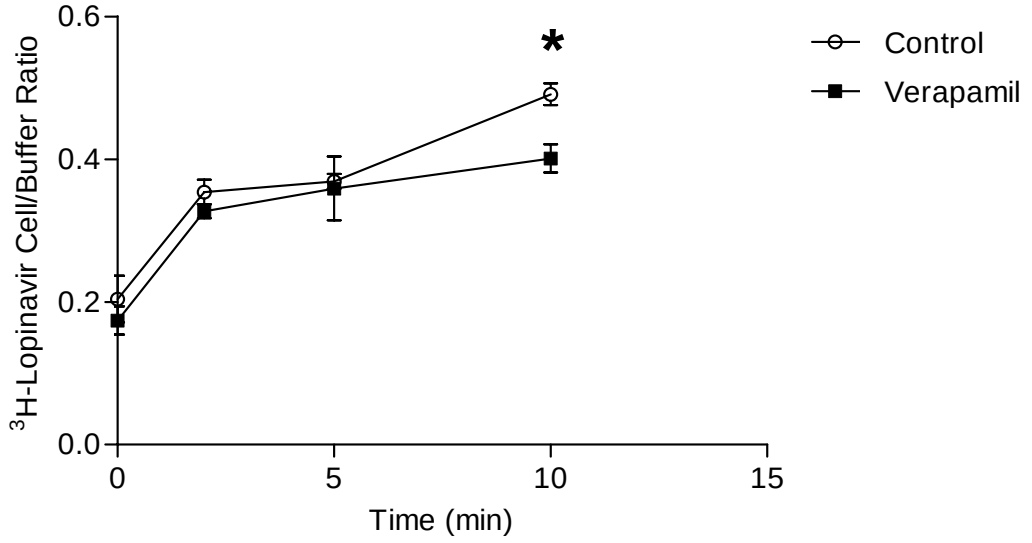
Preliminary uptake and efflux experiments on standard cell culture plates were carried out with ^{3}H-lopinavir (0.32 μM) and different inhibitors of ABC transporters present on the maternal side of the placental syncytiotrophoblast.

There were some technical issues that came up and thus, hindered interpretation of results. The experiments with the results below had one or more of the following issues:

1. Cells were not washed with ice-cold buffer before adding the lysis buffer in an uptake experiment.
2. In an efflux experiment, cells were not washed with ice-cold buffer after aspirating the uptake buffer and before adding the efflux buffer. They were also not washed before adding the lysis buffer.
3. Stock buffers were not analyzed
4. Samples from stock solutions of ^{3}H-lopinavir ± verapamil were not counted in triplicate, so as to account for the difference in ^{3}H-lopinavir in the two stocks

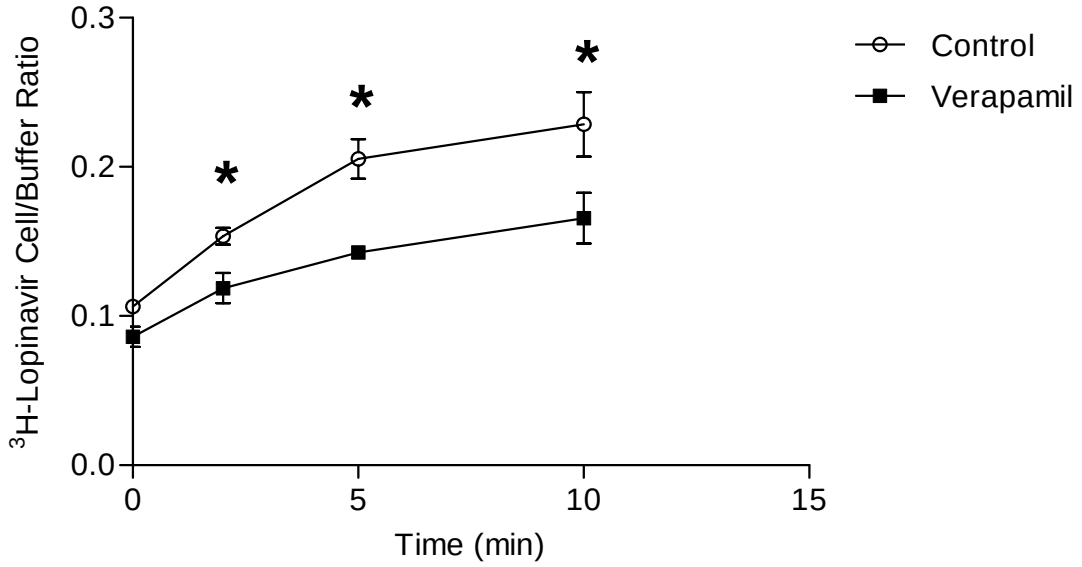
An uptake experiment with BeWo cells grown on 12-well plates was done with ^{3}H-lopinavir (0.32 μM). Verapamil (100 μM) was used as an ABCB1 inhibitor. A significantly lower ^{3}H-lopinavir cell/buffer ratio was observed in the presence of verapamil at 10 minutes (Figure 14). The results suggested that uptake of ^{3}H-lopinavir was inhibited in the presence of verapamil. This was not consistent with ABC transporters but suggested an involvement of uptake transporters.

Figure 14: Uptake of ^{3}H-lopinavir (0.32 μM) in the presence or absence of verapamil (100μM). Results are presented as mean ± standard deviation and were analyzed using two-way ANOVA followed by Bonferroni post-tests pairwise comparisons. Asterisks indicate significant difference ($p < 0.05$); other comparisons were not significant.



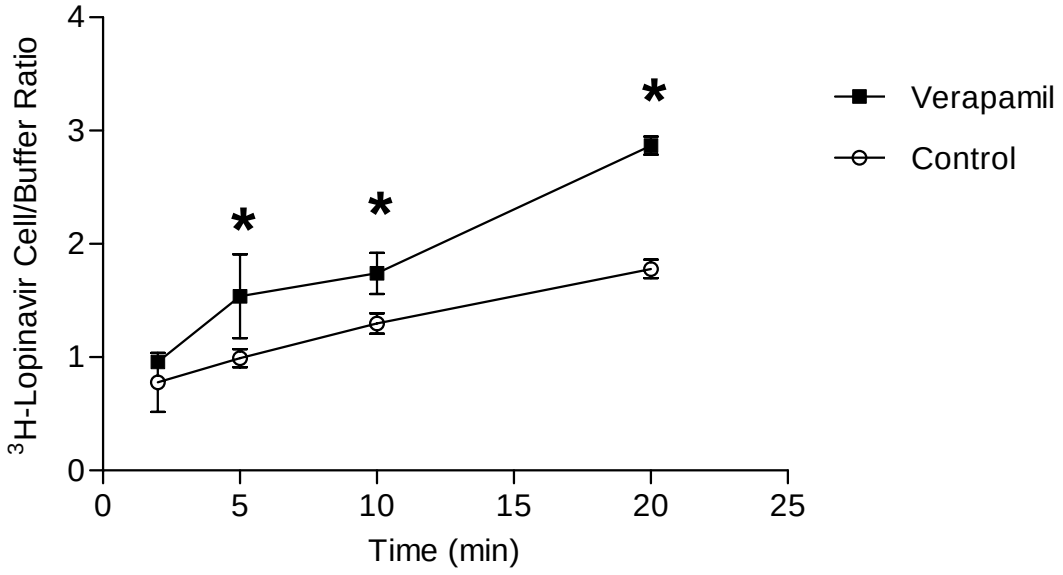
The above experiment was repeated with two washing steps before adding the lysis buffer (Figure 15). A significantly lower ^{3}H-lopinavir cell/buffer ratio was again observed in the presence of verapamil, at all time intervals studied. Verapamil inhibited apparent ^{3}H-lopinavir uptake by ~30% in 10 minutes. The results were consistent with inhibition of uptake transporters or stimulation of ABC transporters, and inconsistent with inhibition of ABC transporters, as originally expected.

Figure 15: Uptake of ^{3}H-lopinavir (0.32 μM) in the presence or absence of verapamil (100μM). Results are presented as mean ± standard deviation and were analyzed using two-way ANOVA followed by Bonferroni post-tests pairwise comparisons. Asterisks indicate significant difference ($p < 0.05$); other comparisons were not significant.



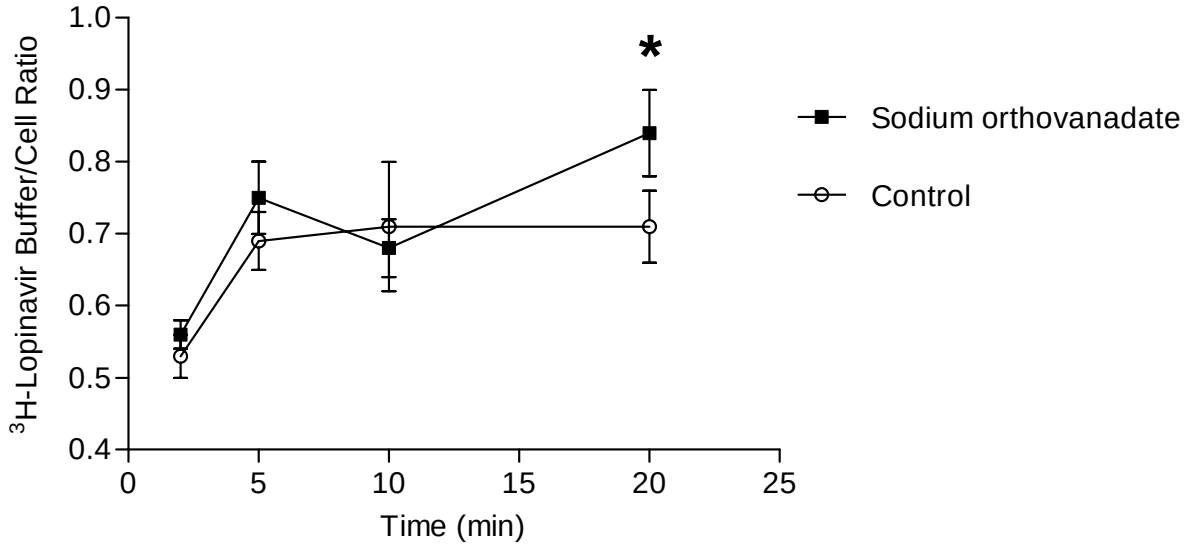
An efflux experiment done with ^{3}H-lopinavir and verapamil showed a significantly higher buffer/cell ratio in the presence of verapamil at 5, 10, and 20 minutes (Figure 17). Lopinavir efflux was linear with time to 20 minutes and the efflux rate (slope) was increased by verapamil. The results again indicated an inhibition of uptake by verapamil or a possible stimulation of efflux by verapamil.

Figure 16: Efflux of ^{3}H-lopinavir (0.32 μM) in the presence or absence of verapamil (100μM). Results are presented as mean ± standard deviation and were analyzed using two-way ANOVA followed by Bonferroni post-tests pairwise comparisons. Asterisks indicate significant difference ($p < 0.05$); other comparisons were not significant.



An efflux experiment was also done with ^{3}H-lopinavir and sodium orthovanadate (500 μM) as an inhibitor of all ATP-dependent processes (Figure 18). A significantly higher ^{3}H-lopinavir buffer/cell ratio was observed in the presence of sodium orthovanadate, which indicated an increase in ^{3}H-lopinavir efflux in the presence of sodium orthovanadate. The cells were, however, not washed before adding the lysis buffer and the results were, therefore, again inconclusive.

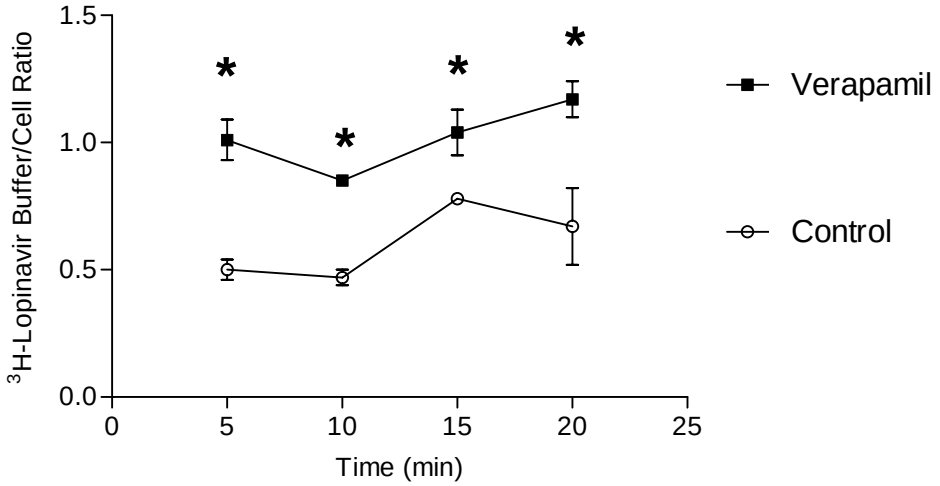
Figure 17: Efflux of ^{3}H-lopinavir (0.32 μM) in the presence or absence of sodium orthovanadate (500μM). Results are presented as mean ± standard deviation and were analyzed using two-way ANOVA followed by Bonferroni post-tests pairwise comparisons. Asterisks indicate significant difference ($p < 0.05$); other comparisons were not significant.



Triplicate samples from the stock solutions of ^{3}H-lopinavir ± sodium orthovanadate in the above experiment were counted and the results indicated that there was a significant difference between ^{3}H-lopinavir present in the two stocks; the stock with sodium orthovanadate having 3-times higher ^{3}H-lopinavir concentration compared to the stock without sodium orthovanadate.

An efflux experiment was carried out with a pre-incubation step in the presence of 0.05 % BSA so that BSA occupies the non-specific binding sites and a better estimate of lopinavir transport can be determined. A significantly higher ^{3}H-lopinavir buffer/cell ratio was observed in the presence of verapamil at 5, 10, 15 and 20 minutes (Figure 19). This again showed that verapamil either inhibited ^{3}H-lopinavir uptake or stimulated its efflux.

Figure 18: Efflux of ^{3}H-lopinavir (0.32 μM) in the presence or absence of verapamil (100μM). Results are presented as mean ± standard deviation and were analyzed using two-way ANOVA followed by Bonferroni post-tests pairwise comparisons. Asterisks indicate significant difference ($p < 0.05$).

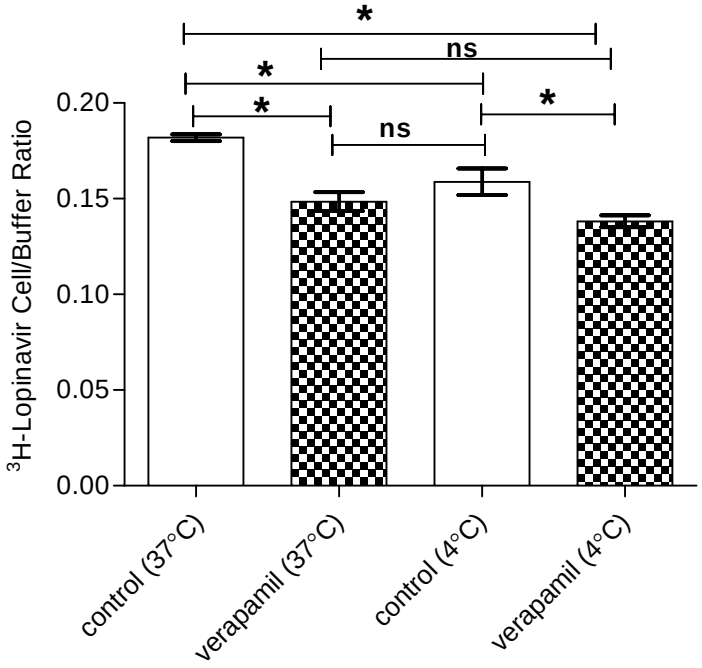


Verapamil may also inhibit a bi-directional uptake transporter present on the apical side of the BeWo cells. Because verapamil was observed to displace ^{3}H-lopinavir from non-specific binding sites, a possibility of it displacing ^{3}H-lopinavir from intracellular binding sites and resulting in an increase in unbound ^{3}H-lopinavir fraction in the cells and therefore, causing an increase in efflux cannot be ruled out. To further solve this puzzle, it was necessary to differentiate between the uptake and efflux processes by not including verapamil at the uptake step in the efflux experiments, in contrast to the previous experiments where verapamil was present at the uptake step in the efflux experiments.

Experiments to differentiate between uptake and binding:

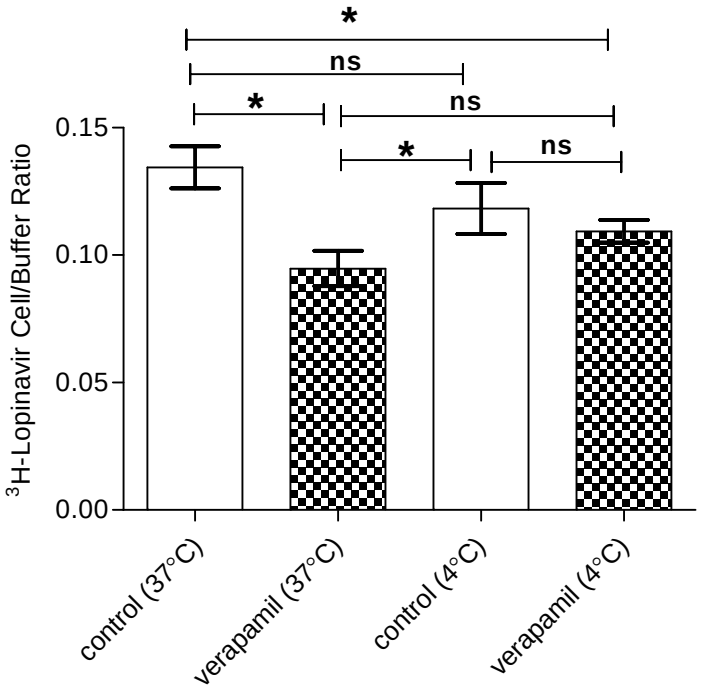
To differentiate between binding and uptake issues, further uptake and efflux experiments were carried out at 37°C and at a lower temperature (4°C), assuming that the transport processes at 4°C would be inhibited and therefore, would help to differentiate between uptake and binding processes. The experiments were carried out as mentioned above, but at two temperatures with control and verapamil treated group. There were a few issues that came up with the initial experiments and those results are shown in Figures 19-21 and discussed below:

Figure 19: Uptake experiment with ^{3}H-lopinavir (0.32 μM) and verapamil (100 μM) at 37°C and 4°C. Results are presented as mean ± standard deviation and were analyzed using two-way ANOVA followed by Bonferroni post-tests pairwise comparisons. Asterisks indicate significant difference ($p < 0.05$); "ns" indicates that the comparison was not significant.



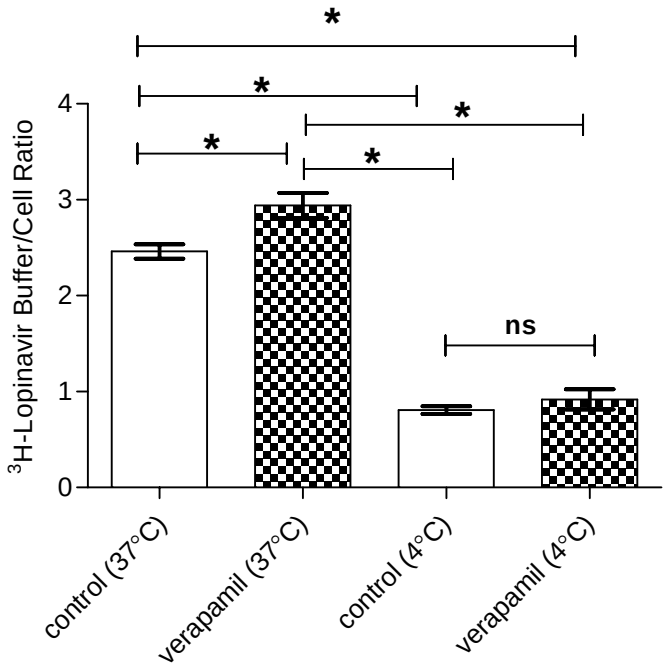
There was no washing done with ice-cold buffer (4°C) before adding the lysis buffer.

Figure 20: Uptake experiment with ^{3}H-lopinavir (0.32 μM) and verapamil (100 μM) at 37°C and 4°C. Results are presented as mean ± standard deviation and were analyzed using two-way ANOVA followed by Bonferroni post-tests pairwise comparisons. Asterisks indicate significant difference ($p < 0.05$); "ns" indicates that the comparison was not significant.



The uptake step at 4°C was not exactly 10 minutes.

Figure 21: Efflux experiment with ^{3}H-lopinavir (0.32 μM) and verapamil (100 μM). Results are presented as mean ± standard deviation and were analyzed using two-way ANOVA followed by Bonferroni post-tests pairwise comparisons. Asterisks indicate significant difference ($p < 0.05$); "ns" indicates that the comparison was not significant.



Washing was done with buffer at 37°C for the experiment at 37°C. Also, the uptake step at 4°C for 10 minutes was actually at 37°C for 5 minutes and at 4°C for further 5 minutes.

VITA

Abhishek Gulati was born on August 25, 1980 in Delhi, India. He received a Bachelor of Pharmacy from Faculty of Pharmacy, Jamia Hamdard University, Delhi, India in 2003. He then received a Master of Pharmacy (Pharmaceutics) from Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala, Punjab, India in 2005. He has authored 2 posters at national meetings and has published 2 peer reviewed publications related to his graduate research.